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AMILTON B. G. ROBINSON

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EDITOR'S VIEWPOINT

That finger- and thumb-sucking produce changes in the relationship of the teeth and jaws has been generally accepted by most orthodontists, yet the finger-sucking problem has remained a subject for controversy when correction of the habit is considered. As graduate students in orthodontics, Ruttle, Quigley, Crouch, and Ewan (p. 739) studied seriatim dental records (annual orthodontic casts) of thirty-six thumb- and finger-suckers. Comparing these with non-finger-suckers, it was shown that the habit results in elongation of the anterior segment of the maxillary arch and an opening (overjet) in the anterior region. As might be expected, the elongating effect in the maxilla opposes any tendency to incisor crowding in that region. These data do not indicate that thumb- or finger-sucking has any significant effect on molar occlusion. This commendable investigation illuminates an area of clinical conjecture and illustrates the value of studies on carefully recorded clinical material.

Lefkowitz, Bodecker, and Mardfin (p. 749) present a detailed description of odontogenesis of the rat molar in the prenatal period. This well-illustrated contribution is an atlas of rat molar development and should be of great value to those interested in normal and abnormal odontogenesis. The authors credit a group of cells at the base of the oral mucosa with the initiation of tooth formation; they ascribe little function to the outer enamel epithelium (which they prefer to call the outer tunic) or to the stellate reticulum. They propose that the stratum intermedium may exert an organizer effect on the preameloblasts, and also assign an organizer role to the active preameloblasts. They observed transformation of fibroblasts into odontoblasts. This study in minutia was long overdue.

Shklar and Glickman (p. 773) elicited the alarm reaction in rats as evidenced by changes in the adrenals, thymus, spleen, and gastrointestinal tract. Changes were also noted in the salivary glands but, surprisingly, the periodontium was not altered. This early study on the effect of the alarm reaction on the periodontium will no doubt be followed by others, and perhaps it will be shown that the period of production may need to be longer, the period of maintenance may need to be increased, or that the alarm reaction may only condition the periodontium for other factors.

Histochemical studies by Engel (p. 779) on tissue from normal and inflamed gingiva show a definite increase in water-soluble mucoprotein carbohydrates and loss of definition of the basement membrane. As Engel points out, bacterial enzymes may bring about such changes, or they may result from intrinsic factors such as the hormonal disequilibrium in pregnancy or puberty. Gingival disease is still not well defined, and this type of basic study is needed to help overcome traditional prejudice.

Anderson (p. 785) describes a small strain gauge, imbedded in an inlay, for accurate analysis of chewing forces. While this apparatus has obvious limitations, it offers a method for careful analysis of chewing under various conditions.

Curby and Wuehrmann (p. 790), using an experimental approach, attempted to determine whether standard kilovolt peak and milliampere seconds could be employed to secure adequate intraoral radiographs. Their study suggests that 95 KVP affords a greater range of tissue densities observable in the radiograph at a constant milliampere second than any other kilovolt peak from 50 to 110. At a 23 inch target distance 10 to 15 Ma. seconds, 0.5 mm. Al filtration and ultraspeed film they recommend 95 KVP as optimum. A technic which uses standardized kilovolt peak and milliampere second is highly desirable, clinically.

Shafer (p. 796) applied fractionated doses of x-ray irradiation to the major salivary glands of the rat. While marked inflammation and degenerations were noted with

both single and fractionated doses, it was felt that the histologic changes were not of the irreversible type. Since observations on patients who have received heavy doses of irradiation over the salivary glands suggest that there are marked alterations in function, the author suggests that the utilization of histochemical technics might give information which would point out physiologic alterations and possible histologic alterations which are not observable with the technics used.

Radike and Muhler (p. 807) placed 10 mg. per milliliter of sodium fluoride and stannous fluoride in the water of hamsters fed cariogenic diets. The stannous fluoride was substantially more effective than sodium fluoride in reducing cavity production, confirming an observation in rats. Stannous fluoride was stored at a lower level in bone than was sodium fluoride, a finding throwing doubt on the validity of an earlier statement that caries reduction parallels bone storage. The authors use "severity index" as a measure of caries activity. There is great need for standardized indices of caries activity in man and experimental animals. Such a project is being undertaken under auspices of the American Dental Association's Council on Dental Therapeutics. We wish them success and hope that their findings will be universally accepted.

Dunning (p. 811) has analyzed available data from this country and others in an effort to determine whether certain geographic considerations influence dental caries occurrence. His discussions are interesting and frank. He admits that his statistical studies suggest probabilities and that common sense is essential in consideration of the data and the mathematical results. The suggestive pattern that caries increased with latitude and distance from the seacoast is challenging and worthy of further study for the "why." As Dunning points out, geographic factors may influence not only exposure to sunshine and fluorine but even dietary patterns.

Besic (p. 830) has continued his efforts to produce bacteria-free carious lesions in vitro. He illustrates carieslike lesions produced by the action of various acid solutions with calcium and phosphorus ions in less than maximal concentration. This does not appear remarkable to us, and we believe that the lesions might be reproducible by any acid applied intermittently to a gelatinous mass on the tooth surface. This would reproduce more nearly to the conditions attendant upon caries decalcification in vitro.

Kauffman, Kasai, and Koser (p. 840) found the amounts of folic acid and vitamin B_6 in the saliva adequate for maximal or near maximal growth of lactic bacilli.

Ericsson (p. 850) investigated the occurrence of citric acid in the saliva. He found that while the citric content of saliva increased after ingestion of citrate-rich materials, this salivary concentration rapidly decreased. The citrate concentrations occurring two minutes after ingestion had no apparent effect on the solubility of the enamel. From a practical standpoint, this would indicate that individuals showing erosion of the teeth as a result of unusual consumption of citrate-rich fruits and beverages produced the undesirable effects chiefly at the time during which the citrate-rich material was being consumed. Apparently the saliva can rapidly clear the citrate, as well as have a buffering effect. The importance of citric-containing fruits and beverages as causes of erosion of human teeth has probably been overemphasized, and it is the rare individual with unusual eating habits who shows the effects, rather than the average individual.

Nikiforuk and Sreebny (p. 859) have investigated a chelating agent, ethylenediamine tetracetic acid (Edt), as a demineralizer of bones and teeth. The peculiar advantage of Edt is its ability to remove calcium salts at a neutral pH, thereby avoiding denaturation of proteins. While it takes about seven times as long to demineralize bones in Edt as in acids, the method offers many advantages in preserving the organic portion of enamel for histologic and chemical studies.

Keyes (p. 868) describes a watering system for small animals in the laboratory. Such information may save much work by other investigators.

H. B. G. R.

A SERIAL STUDY OF THE EFFECTS OF FINGER-SUCKING

ALLAN T. RUTTLE, D.D.S., M.S., WILLIAM QUIGLEY, D.D.S., M.S., JOHN T. CROUCH, D.D.S., M.S., AND GEORGE E. EWAN, D.D.S., M.S.*

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ORTHODONTISTS have long been interested in the distorting effects of finger habits upon the denture. With little detailed study and with little collected evidence, clinicians have developed several, often contradictory, viewpoints about the probable and actual effects of these finger habits. Since finger habits are widely practiced by young children, for short or long periods of time, it is important to ascertain the impact of these habits upon the denture and to establish their contribution to the etiology of malocclusion. The serial orthodontic models in the laboratory of child development at the University of Michigan are sufficiently extensive to begin a systematic attack upon this problem.

DATA

Since 1931, systematic seriatim records on child development have been taken at the University of Michigan, the university conducting a thorough growth study of all its students. Included are psychological, educational, growth, medical, and dental records. The dental history consists of annual orthodontic models and a biannual oral examination. All observations for this study were taken from the orthodontic casts. Serial records of various lengths for some 1,700 children have been compiled to date.

Thirty-six children, who have long records, present definite and persistent oral habits. The detailed distribution of these data is recorded in Table I. Thumb-sucking predominates, occurring in twenty-three instances, with finger-sucking limited to three children, and such compound oral habits as thumb-sucking, finger-sucking, and nail-biting accounting for the remaining ten individuals. All habits, except one, started during the first year of life. The age at which the first cast was taken appears in Column 3 (Table I), and the age at which the last habit record was taken appears in Column 4. The age at the last model, the length of the record, the number of casts for each child, and the length of time that the habit was operating are shown in the remaining columns of the table. Seven cases received orthodontic treatment at various ages. It was necessary to eliminate those casts immediately following the initiation of treatment. These are recorded as treated.

The independent theses submitted in partial fulfillment of requirements for the degree of Master of Science in Orthodontics at the University of Michigan are rewritten jointly by the four authors for publication.

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*Present address: Sheridan, Wyo.

TABLE I

DISTRIBUTION OF TYPE AND DURATION OF HABIT IN MONTHS

CASE NUMBER	TYPE OF HABIT*	AGE AT FIRST CAST	AGE WHEN LAST OBSERVED†	AGE AT LAST CAST	LENGTH OF RECORD	NUMBER OF CASTS	LENGTH OF HABIT RECORD
1	TS	66	96 Stopped	198	132	11	30
2	TS	79	121 Stopped	191	112	9	42
3	FS	56	107 Stopped	205	149	11	51
4	TS	40	97 Treated	97	57	5	57
5	TS	80	113 Stopped	213	133	9	33
6	TS and FS	49	122 Stopped	186	137	11	73
7	FS	76	123 Stopped	220	144	12	47
8	TS	42	97 Treated	97	55	6	55
9	FS and TT	43	84 Treated	67	24	3	41
10	TS	39	84 Stopped	120	81	7	45
11	TS	48	48 Continuing	48		1	
12	TS	43	90 Continuing	90	47	4	47
13	TS and TT	73	98 Continuing	109	36	4	25
14	TS	60	84 Continuing	84	24	3	24
15	TS	44	72 Continuing	72	28	4	28
16	TS, FS and TT	64	135 Stopped	182	118	11	71
17	TS and FS	96	120 Treated	120	24	3	24
18	TS and NB	60	100 Stopped	192	132	6	40
19	FS	34	84 Stopped	121	87	8	50
20	TS and FS	69	138 Treated	138	69	7	69
21	TS	60	142 Stopped	153	93	8	82
22	TS	79	198 Continuing	198	119	11	119
23	TS	77	122 Stopped	198	121	9	45
24	TS	74	165 Treated	165	91	5	91
25	TS and FS	60	84 Stopped	192	132	9	24
26	TS	52	102 Stopped	196	144	13	50
27	TS and TT	84	108 Continuing	108	24	3	24
28	TS	60	108 Continuing	117	57	4	48
29	TS	108	108 Continuing	108		1	
30	TS	41	54 Continuing	54	13	2	13
31	TS	91	138 Continuing	138	47	4	47
32	TS	42	90 Continuing	99	57	5	48
33	TS	60	84 Continuing	94	34	4	24
34	TS and TT	54	114 Continuing	116	62	6	60
35	TS	60	106 Continuing	106	46	5	46
36	TS	54	81 Continuing	81	27	3	27

*TS, Thumb-sucking; TT, tongue-thrusting; FS, finger-sucking; NB, nail-biting.

†All habits commenced during the first year of life, with the exception of Case 24, which started at 30 months of age.

A comparable set of observations were secured upon forty-two nonhabit cases. This evidence is presented in Table II.

OBSERVATIONS

Arch Form.—Arch form was classified as tapering, trapezoid, and ovoid since other arch types were not observed. A tapering arch (Fig. 1) converges from the molars to the first incisors to such an extent that lines passing through the central grooves of the molars and premolars intersect within 1 inch anterior to the first incisors. A trapezoidal arch (Fig. 2) shows the same type of convergence toward the midline as the tapering arch, but to a lesser

TABLE II

DISTRIBUTION OF NONHABIT CASES AND DURATION IN MONTHS

CASE NUMBER	AGE AT FIRST CAST	AGE AT LAST CAST	LENGTH OF RECORD	NUMBER OF CASTS
1	59	112	53	6
2	74	169	95	9
3	76	137	61	6
4	67	134	67	7
5	85	198	113	9
6	100	196	96	9
7	63	194	131	10
8	71	203	132	12
9	58	153	95	8
10	122	223	101	9
11	37	191	154	12
12	42	131	89	6
13	72	206	134	8
14	53	196	143	12
15	75	159	84	8
16	73	215	142	12
17	64	194	130	11
18	38	169	131	12
19	72	205	133	12
20	65	208	143	12
21	87	185	98	9
22	46	156	110	9
23	96	200	104	10
24	90	132	42	4
25	64	146	82	8
26	64	156	92	10
27	84	102	18	2
28	55	208	153	14
29	46	213	167	13
30	49	215	166	14
31	69	122	53	5
32	41	172	131	10
33	77	147	70	7
34	74	209	135	9
35	44	185	141	12
36	62	166	104	9
37	54	126	72	7
38	39	121	82	8
39	43	186	143	10
40	43	209	166	15
41	42	183	141	13
42	47	184	137	12

degree. The anterior teeth are somewhat squared to abruptly rounded from canine tip to canine tip. The canine teeth act as corners of the arch. Ovoid arches (Fig. 3) curve continuously from the molars of one side to the molars of the opposite side in such a way that two such arches placed back to back describe an oval.

Both maxillary and mandibular arches were observed and tabulated according to the requirements just mentioned. The depth of bite, true overjet, apparent overjet, anterior spacing, anterior crowding, and arch widths for maxillary and mandibular deciduous and permanent canines, deciduous molars and premolars, and first permanent molars were measured by the use of the Boley or Helios gauge, the choice being that which best fitted the observation.

Depth of Bite.—The depth of bite was determined by measuring the average overlapping of the labial surfaces of the mandibular first incisors by the

lingual surfaces of the maxillary first incisors. This measurement was made vertically on the mandibular first incisors from the incisal edge to a point on the labial surface in line with the maxillary incisal edges and parallel to the occlusal plane. The degree of open-bite is the average distance from the incisal edges of the maxillary first incisors to the incisal edges of the mandibular first incisors, measured vertically and perpendicular to the occlusal plane. These measurements on depth of bite and open-bite were not taken if the maxillary and mandibular first incisors were less than two-thirds erupted, and in cases where edentulous spaces existed.

Fig. 1.

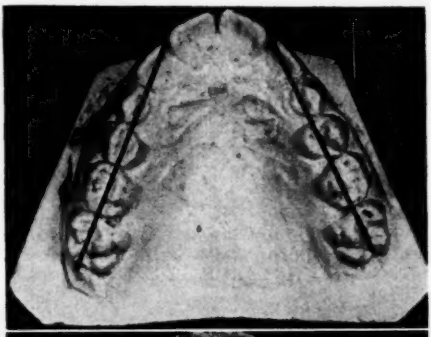

Fig. 2.

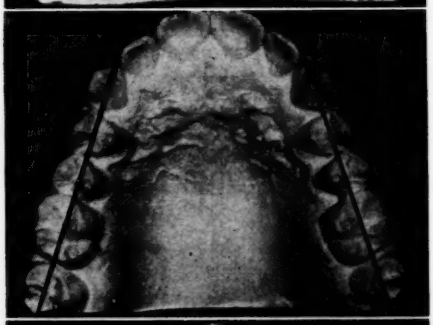

Fig. 3.

True Overjet.—The grade of overjet is listed as zero when the incisal edges of the mandibular first incisors contact the lingual surfaces of the maxillary first incisors just incisal to the cervical prominences, or cingula. Deviations from this state in an anterior or posterior direction and parallel to the occlusal plane are recorded in millimeters.

Apparent Overjet.—Apparent overjet is the distance from the labial surface of the mandibular first incisors to the labial-incisal angle of the maxillary first incisors measured parallel to the occlusal plane.

Anterior Spacing.—Anterior spacing was measured by placing the arms of the Boley gauge perpendicular to the lateral edges of the space. The total amount of space, secured by adding the individual measurements, was recorded.

Canine Width.—The canine width measured on the maxillary and mandibular deciduous and permanent canines is the greatest dimension between the labial surfaces of the respective canines.

First Molar Arch Width.—The first molar arch width is the maximum diameter between the buccal surfaces at the gingival line. It was recorded for permanent and deciduous teeth.

First Premolar Arch Width.—The first and second premolar arch width was obtained by measuring the distance between the most marked convexities of the buccal tooth surfaces. Maxillary and mandibular measurements were recorded.

Deciduous Second Molar Arch Width.—The maxillary and mandibular deciduous second molar arch width is the distance between points where extensions of the buccal grooves intersect with the gingival line.

Classification of Molar Occlusion.—The Angle classification of occlusion was used for the first permanent molars. An equivalent system was used for the second deciduous molars, which were used for this classification as long as they were present. Variations from Class I (Angle) are indicated in terms of quarters of a cusp.

Classification of Canine Occlusion.—Canine occlusion is described as Class I when the tip of the upper canine interdigitates in the embrasure between the lower canine and first premolar. It is called Class II if the upper canine is mesial to this embrasure, and Class III if it is distal to it. Variation toward Class II or Class III was recorded in quarters of a cusp.

RESULTS

Analysis.—The Chi-square Goodness of Fit* has been used to reduce these data in a way to permit comparison between the habit and the nonhabit groups (Table III). The chance frequency E represents the type of distribution one would obtain if no associative factors were operative. The actual frequency is given as A. The Chi-square value (X^2) is a statistical computation stating whether or not the differences shown are dependent upon real associative factors or whether they have to be ascribed to chance. The sum of the Chi-square values indicates the statistical significance and P is a statement of the chances out of 100 of this Chi-square sum being significant.

*For detailed development and discussion of the Chi-square technic see Peters and Van Voorhis, Statistical Procedures and Their Mathematical Bases, ed. 1, New York, 1940, McGraw-Hill Book Company, Inc., pp. 404-24.

Maxillary Arch Form.—The effect of prolonged finger-sucking upon the maxillary arch is demonstrated in Table III. The habit operates to alter the arch form in such a way as to give a higher frequency of tapered arches and a lower frequency of ovoid arches and no significant change in the frequency of trapezoid arches. This distribution of frequency is assignable to a general tapering of arch form as the effect of the habit, operating to move ovoid into trapezoid and tapering arch forms, and trapezoid to tapering. The differences shown in the table are statistically of high significance, with the probability of ascribing this change to chance being less than 1 in 100 ($P = 0.01$).

TABLE III

MAXILLARY ARCH FORM

	TAPERING	TRAPEZOID	OVOID	TOTAL
Habit	E—3.69 A—6 X^2 1.44	E—28.61 A—30 X^2 0.07	E—3.69 A—0 X^2 3.69	36
Nonhabit	E—4.31 A—2 X^2 1.24	E—33.38 A—32 X^2 0.06	E—4.31 A—8 X^2 3.16	42
Total	8	62	8	78
Sum of X^2 =	9.66	P = 0.01		

Mandibular Arch Form.—The mandibular arch in the habit series presents a more pronounced angulation at the canine and a flattening of the incisors. There is little difference between the labial inclination of the mandibular anteriors in the habit and nonhabit groups. The habit acts to retrude the anterior part of the denture as a whole without changing the inclination of the teeth. This change reduces ovoid and tapering arches and increases trapezoidal arches in the habit group. The change is significant ($P = 0.02$).

Anterior Depth of Bite.—The evidence makes it clear that finger habits operate to produce open-bite malocclusions ($P = 0.01$). The detailed distribution indicates that ten of the thirty-six habit cases are in definite open-bite relationship, whereas in the nonhabit series no open-bite occlusion is shown. The further suggestion, then, is that finger habits presumably represent the major etiological source of this type of malocclusion affecting approximately one-third of all dentures with this habit.

True and Apparent Overjet.—The differences between the habit and nonhabit groups warrant the conclusion that thumb and finger habits produce a major amount of modification in the anterior part of the maxillary arch. The difference in incidence is apparent, with the habit series presenting 45 per cent moderate to extreme true overjet and the nonhabit series showing 9 per cent with true overjet in these categories. The difference between these two percentages shows the habit to produce distortion at the 36 per cent level.

Apparent overjet ($P = 0.01$), like true overjet ($P = 0.01$), is without question another major effect of finger-sucking. Here we find 40 per cent of the habit group and only 5 per cent of the nonhabit group. The marked

difference between the groups at the extreme reveals more clearly the impact of the distortion. Thus, it is realized that true overjet and apparent overjet will occur together many times, although there are some cases in which apparent overjet, due mainly to a labial tipping of the maxillary anteriors, occurs without the development of excessive true overjet. It is questionable, however, that apparent overjet has a higher clinical significance than does true overjet.

Distribution of True and Apparent Overjet.—While the directional distribution of true overjet changes are relatively balanced, apparent overjet shows a definite tendency to increase throughout both the habit (58.8 per cent) and the nonhabit series (52.4 per cent).

The apparent overjet increases independently of any increase in true overjet in 23.5 per cent of the habit cases and in 23.8 per cent of the nonhabit group; conversely, the true overjet increases independently of directional changes in apparent overjet in only one (2.5 per cent) of the habit cases and in no cases of the nonhabit group.

This discrepancy, by careful visual observations, appears to be mainly the result of an increase in labial inclination of the maxillary incisors, with or without a slight lingual movement of the mandibular incisors.

Shift from Anterior Deciduous Spacing to Anterior.—

Permanent crowding: The percentage distribution of transgression from anterior spacing in the deciduous dentition to anterior crowding in the permanent dentition of twenty-seven habit and thirty-nine nonhabit cases is presented. Four cases (14.8 per cent) in the habit series shifted from maxillary anterior spacing of the deciduous teeth to crowding of the maxillary permanent anteriors. In contrast, seventeen cases (43.6 per cent) of the nonhabit series revealed a transition from spacing to crowding of the maxillary anteriors.

In the mandibular arch, there are no particularly significant differences between the habit and nonhabit series with respect to a shift from deciduous anterior spacing to anterior crowding of the permanent teeth. It is evident, then, that oral habits act in such a manner as to retard any inherent tendency for a transition from maxillary deciduous anterior spacing to crowding of the permanent anterior teeth. Habit has no similar effect upon the mandibular anteriors.

Distribution of Progressive Changes in Anterior Crowding.—Three cases (8.8 per cent) of the habit series and fourteen (35.0 per cent) of the nonhabit series show a progressive increase in maxillary anterior crowding. This indicates a greater tendency in the nonhabit cases toward increased crowding in this area. The differences in anterior crowding of the mandibular dentition between the habit and nonhabit groupings are not particularly significant, since ten (29.4 per cent) of the habit series show an increase as compared with fourteen (42.5 per cent) of the nonhabit group showing an increase. That the habit cases show less of a tendency toward maxillary crowding is no doubt

due to a distortion of the anterior part of the arch which is often elongated, (see *True and Apparent Overjet*) with the result that crowding is alleviated and spacing often may develop.

Distribution of Anterior Spacing and Crowding in the Permanent Dentition.—The permanent dentition of the habit group shows a greater tendency toward maxillary anterior spacing than does the nonhabit group. The percentage distribution of these conditions shows eight (47 per cent) of the habit and eleven (35.5 per cent) of the nonhabit series with spacing in this region. Crowding is present in five (29.4 per cent) of the habit cases and in thirteen (41.9 per cent) of the nonhabit cases.

No significant differences, either clinical or statistical, between the two groups are observed in either mandibular spacing or crowding in the permanent dentition.

It is evident, then, that oral habits are conducive to maxillary anterior spacing in the permanent dentition.

Maxillary Arch Width (Canine).—There is no evidence that the deciduous canine arch width in the maxilla and the mandible, or the permanent canine mandibular arch width, is altered by thumb-sucking. Likewise, canine occlusion is unaffected. The Chi-square Goodness of Fit analysis does not make the effects of oral habits particularly clear in the maxillary arch width at the permanent canines. A numerical analysis of amount of change, however, is informative. The average decrease of the twelve nonhabit cases showing a decrease is 0.615 mm., and for those showing an increase it is 1.05 mm. The corresponding averages for the habit group are 1.14 mm. and 0.6 mm., respectively. It appears, therefore, that when maxillary canine arch width is affected by an oral habit the distortion is of a rather marked degree.

Molar Arch Width.—There is no evidence showing deformity in the deciduous molar, premolar, or permanent molar areas of the maxilla or mandible. In the mandibular arch, at the permanent first molar and the deciduous first molar levels, the changes merely approach statistical significance. Conversely, maxillary arch width at the second premolar and first permanent molar levels shows a striking similarity between the habit and nonhabit series.

Molar Occlusion (Angle).—The relationship of the upper and lower molars indicates only an approach toward significance. There is a suggestion that these habits produce a shift toward a Class II molar relationship. The degree of this shift, however, is not marked. Certainly, the contribution of this type of habit to the etiology of Class II malocclusion is not nearly as great as is indicated in the literature. This is in strong contrast to depth of bite, arch form, true overjet, and apparent overjet where the amount of change is large and the clinical significance great.

SUMMARY

The maxillary arch form is modified in thumb and finger suckers by elongation of the anterior segment. This acts to produce spacing, labial inclina-

tion, and protrusion of the maxillary incisors. There is a definite opening action in the anterior region. True and apparent overjet are increased strongly, although apparent overjet shows a tendency to increase in the nonhabit, as well as the habit, series.

Sucking habits act in opposition to any inherent tendency for a transition from maxillary deciduous anterior spacing to crowding of the permanent anterior teeth. This effect is not evident in the mandibular anteriors.

The frequency of distortion of the maxillary arch width at the permanent canine level is limited. In a few cases, however, where a distortion does occur it progresses to a rather marked degree.

Modification of the mandibular arch width at the deciduous first molar and the permanent first molar merely approaches statistical significance. Clinical utility of this fact, however, is questionable.

Maxillary arch width, with the infrequent exception of the permanent canine area, is unaffected.

Contrary to most publications, the effect of thumb and finger habits on the molar occlusion merely approaches statistical significance, and does not attain clinical utility.

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ODONTOGENESIS OF THE RAT MOLAR

Prenatal Stage

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THE rat molar has achieved pre-eminence in the field of dental research and at present supersedes the rat incisor in the exploration of the complexities of development and growth. The rat dentition is monophyodont. The molars are teeth of limited growth. There is no formation of enamel over the tips of the cusps so that upon eruption the dentin is exposed.[1] They have been successfully used in experimentally induced caries,[2, 3] and have provided material for transplantation[4-6] and in vitro studies.[7-11]

A chronological table of normal growth and development would be of inestimable assistance in evaluating the results of transplantation and in vitro studies. This information would provide definite stages of normal growth for planning studies and would establish a yardstick for comparison with experimental development. Furthermore, the feasibility of securing material of known age may help resolve some of the intricacies of dental embryology.

The process of odontogenesis is continuous from the first invagination of the oral mucosa to the completion of the tooth. To facilitate publication, the report is arbitrarily separated into pre- and postnatal stages.

A complete historical review is available in textbooks, and only where specific aspects of this report are related to the literature will references be given.

MATERIALS AND METHODS

The Rockland strain of albino rats was chosen as the experimental animal. A modified Long and Evans technic[12] was used to secure embryos of known age. The time of fertilization was arbitrarily established the morning following copulation. Twenty-four hours later was construed as the first day of embryonic development.[13] Variations in the time of fertilization present no problem in dental embryology because by the ninth day after coitus all embryos tend to achieve the same stage of development.[14] In this study of odontogenesis the many embryos examined showed a remarkable similarity of development for the same age.

One hundred fifty-six embryos were used in the study. All embryo heads were fixed in 10 per cent formalin. Those 16 days and older were decalcified. Serial paraffin sections were cut at from 6 to 10 microns and stained with hematoxylin and eosin, Mallory, Masson, and Wilder. Frontal and sagittal sections were cut so that a three-dimensional picture of the various stages of development and growth could be observed.

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Interpretation of any one section may be misleading. The maxillary dental lamina begins anterior to the mandibular so that after formation of the enamel organ a central section of the maxillary first molar may be observed in the same plane as a section through the mesial cusps of the mandibular. This may give the appearance of an advanced stage of development in either jaw, depending on the plane of the section (Fig. 16). When viewed serially, however, equal development in both jaws is evident.

In the early stages, the dental lamina is an invagination about ten cells thick. When cut obliquely in frontal sections it may appear thicker (Fig. 5). It never occurs in a true vertical position but at an angle to the median line. The mandibular dental lamina is in a lingual relation to the maxillary so that only on rare occasions would both lamina appear in the same sagittal section. It also proved impossible to cut a sagittal section through the entire dental lamina because it curves upon itself.

In the later prenatal stages the dental lamina and the enamel organ appear to change their position, which may be best observed in frontal sections and illustrates the difficulty of obtaining ideal sagittal sections (Fig. 22). Only a fortuitous accident could achieve ideal results. Nevertheless, the observer may visualize the entire depth of the dental lamina and tooth germs in serial sections.

OBSERVATIONS

Histologic examination of the embryo began at 10 days. At 12 days the oral groove was observed. At 13 days there is a change in the oral mucosa in the area of the future ridges. At 14 days the beginning of lamina formation is present. This report will describe the development of the first molar of the rat at 24-hour intervals from 13 days in utero to birth.

Thirteen Days.—At 13 days the mucosa of the nasal-oral cavity consists of simple cuboidal epithelium except in the area of the future ridges. Here the epithelium is stratified and consists of undifferentiated cells. The accumulation of cells is from without inward and does not indicate lamina formation (Fig. 1).

Fourteen Days.—In the area of the future ridges there is an invagination of epithelial cells arising from the germinal layer of the oral mucosa (Fig. 2). This is the first manifestation of lamina formation. The cells at the fundus of the invagination are undifferentiated and show mitosis (Fig. 3). This is the proliferative zone which will migrate from the oral mucosa to the apex of the tooth outlining the periphery of the dentin. It also gives rise to all the cells of the enamel organ. The germinal cells of the oral mucosa are cuboidal in the area of the future ridges. Proliferation of fibroblasts occurs simultaneously around the dental lamina. In sagittal section, the invagination extends along the entire future ridges (Fig. 4).

Fifteen Days.—Growth has occurred by further invagination of epithelium into the mesenchyme (Fig. 5). The lamina presents the proliferative zone in the outer cells of the fundus (Fig. 6). The remaining peripheral cells are columnar. The epithelial cells occupying the lamina are undifferentiated and as

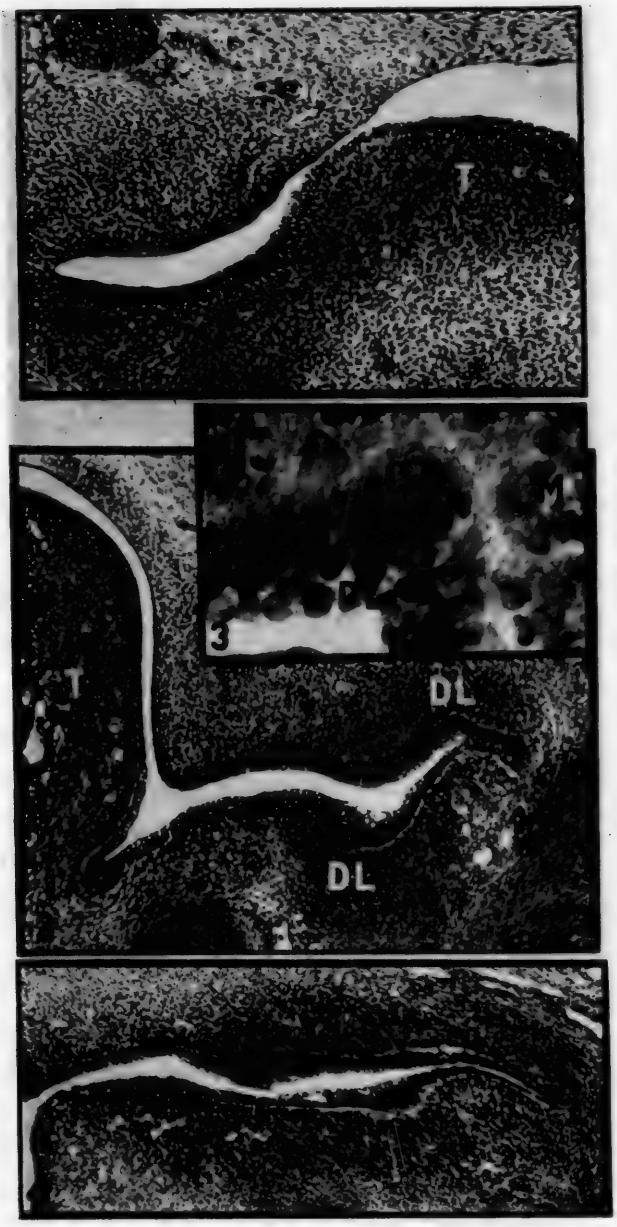


Fig. 1.—Thirteen-day rat embryo. Frontal section. *T*, Tongue. (Original magnification, ×150; reduced ⅓.)

Fig. 2.—Fourteen-day rat embryo. Frontal section. *DL*, Dental lamina; *T*, Tongue. (Original magnification, ×150; reduced ⅓.)

Fig. 3.—Fourteen-day rat embryo. *DL*, Dental lamina (note cell division); *M*, mesenchyme, showing cell division. (Original magnification, ×1200; reduced ⅓.)

Fig. 4.—Fourteen-day rat embryo. Sagittal section. Dental lamina extending entire length of future ridge.

they approach the surface of the oral epithelium become chromophobic, similar to the surface cells of the oral epithelium (Fig. 5). The lamina approaches from the buccal surface and invaginates lingually. The proliferation of fibroblasts continues around the dental lamina and budding capillaries are evident at the periphery.

The tongue almost completely occupies the combined oral and nasal cavity. An epithelial ingrowth or band may be observed mesial to the dental lamina which will divide it to establish the palatine process (Fig. 5). In sagittal section the dental lamina extends along the entire future ridges.

Development during the fourteenth and fifteenth days may be described as the lamina stage.

Sixteen Days.—Growth is manifested by a widening of the fundus of the lamina. The epithelial invagination assumes the configuration of an Erlenmeyer flask with the neck toward the oral mucosa (Fig. 7). The first-formed part of the lamina retains its orginal morphology, and will be referred to as the neck of the enamel organ. This is not a constriction, but appears so because the deeper portion, designated the body of the enamel organ, grows peripherally.

The oral mucosa is unchanged. The outer layer of the oral epithelium is continuous over the dental lamina. No basal layer exists in the area of the neck. The dividing cells are now found in the walls of the fundus (Fig. 8), and growth occurs peripherally with a slight invagination so that a concavity is formed at the base. Greater growth occurs on the lingual, so that even though the lamina approaches from the buccal its base is practically horizontal. The cells occupying the body are mainly round undifferentiated epithelium becoming more chromophobic from without inward.

The condensation of proliferating fibroblasts continues around the flasklike lamina, the greatest density occurring at the base (Fig. 7). The cells are larger and stain more deeply than the outlying mesenchyme. Increased vascularity may be observed at the periphery of the condensation.

Medial growth of the future palatine process continues although fusion is incomplete (Fig. 7). Bone formation appears in the mandible and lies inferiorly and buccally to Meckel's cartilage. In the maxilla, it descends from above buccal to the invaginating epithelium.

When viewed in sagittal section (Fig. 9) the dental lamina extends the entire length of the future ridges which show evidence of anteroposterior growth.

Seventeen Days.—Growth of the body of the enamel organ has progressed peripherally with slight invagination. Greater growth is evident lingually. In frontal section it has the general appearance of a crescent attached to the oral mucosa by the neck. The inclination of the neck toward the lingual is caused by the growth of the ridges (Fig. 10).

The oral mucosa presents a germinal layer in isolated areas. The superficial layer is two to three cells thick and consists of either squamous or round epithelium. The histologic and morphologic appearance in the neck of the enamel organ remains the same.

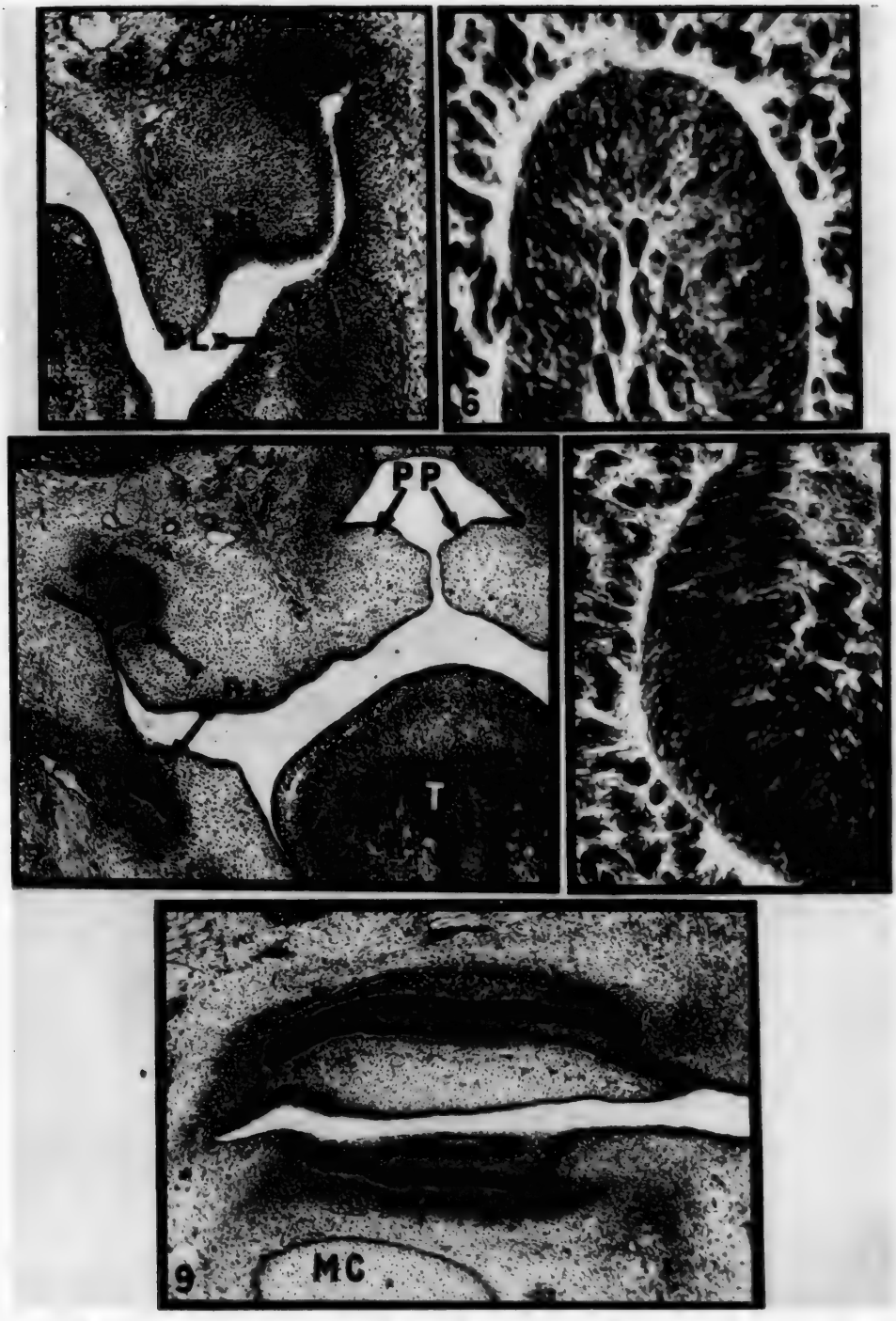


Fig. 5.—Fifteen-day rat embryo. Frontal section. *DL*, Dental lamina; *T*, tongue; *PB*, palatine band. (Original magnification, ×120; reduced ⅖.)

Fig. 6.—Fifteen-day rat embryo. Proliferative zone. Note cell division. (Original magnification, ×1200; reduced ⅖.)

Fig. 7.—Sixteen-day rat embryo. Frontal section. *DL*, Dental lamina; *PP*, palatine process; T, tongue. (Original magnification, ×120; reduced ⅖.)

Fig. 8.—Sixteen-day rat embryo. Proliferative zone. Note cell division. (Original magnification, ×1200; reduced ⅖.)

Fig. 9.—Sixteen-day rat embryo. Sagittal section. Dental lamina extending the entire length of the future ridge. *MC*, Meckel's cartilage.

The body of the enamel organ may now be divided into three groups of cells. (Diagram 1.) The core of the enamel organ known as the stellate reticulum will be referred to as the central cells. They are undifferentiated round epithelial cells which now show microvacuoles in the cytoplasm. The presence of vacuoles gives these cells their stellate appearance (Fig. 11). Proceeding peripherally the cells appear more viable and form a layer around the entire body of the enamel organ. These are the cells of the middle layer, which, in the area of the preameloblasts, will become the stratum intermedium (Fig. 11).

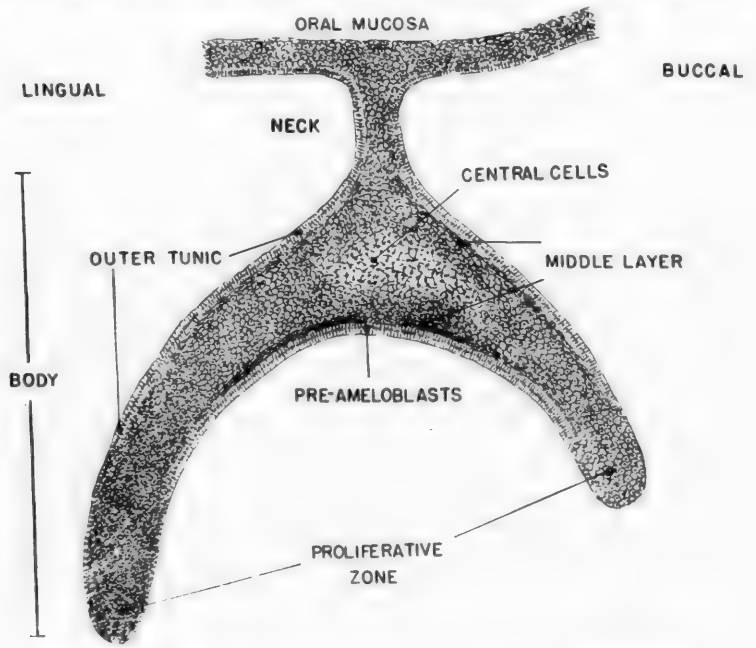


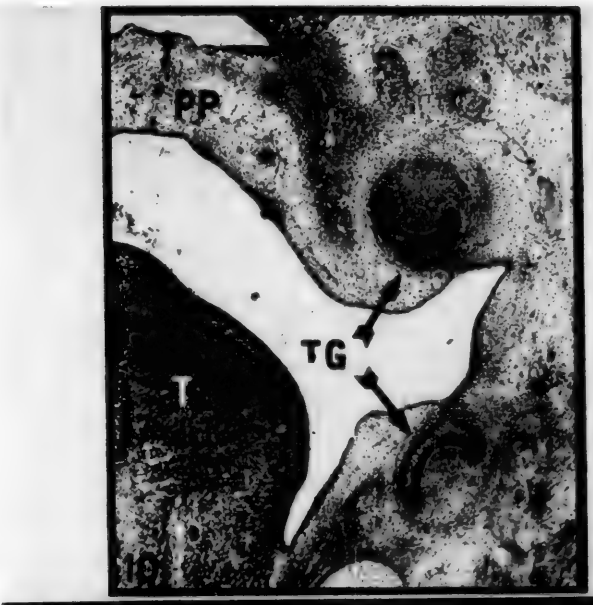
Diagram 1.—Seventeen-day rat embryo. Elements of enamel organ.

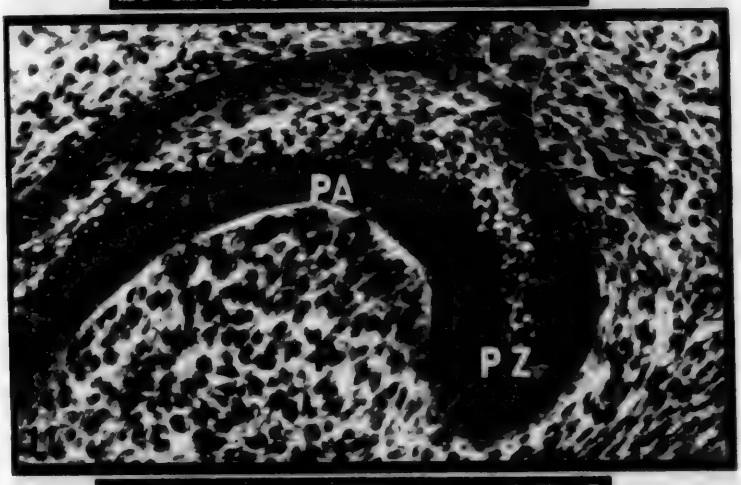
The peripheral layer of the enamel organ exhibits three types of epithelium. The outer enamel epithelium, which will be referred to as the outer tunic, consists of columnar cells (Fig. 11). In the area of the proliferative zone there is undifferentiated epithelium where active mitosis may be observed (Figs. 11 and 12). In the concave base of the body the cells have differentiated into columnar epithelium with centrally located nuclei. They are slightly longer than the columnar epithelium of the neck and outer tunic (Fig. 11). These are the preameloblasts, which will differentiate into ameloblasts after birth.

Fig. 10.—Seventeen-day rat embryo. Frontal section. *TG*, Tooth germ; *PP*, palatine process; *T*, tongue. (Original magnification, ×75; reduced ⅔.)

Fig. 11.—Seventeen-day rat embryo. *N*, Neck; *OT*, outer tunic; *ML*, middle layer; *CC*, central cells; *PA*, preameloblasts; *PZ*, proliferative zone; *P*, papilla. (Original magnification, ×550; reduced ⅔.)

Fig. 12.—Seventeen-day rat embryo. Proliferative zone. Note cell division. (Original magnification, ×1200; reduced ⅔.)





Figs. 10 to 12. *(For legends, see opposite page.)*

Fig. 13.—Eighteen-day rat embryo. Frontal section. *TG*, Tooth germ; *PP*, palatine process; *T*, tongue. (Original magnification, ×85; reduced ⅔.)

Fig. 14.—Eighteen-day rat embryo. *CC*, Central cells; *OT*, outer tunic; *ML*, middle layer; *PA*, preameloblasts; *P*, papilla. (Original magnification, ×550; reduced ⅔.)

Fig. 15.—Eighteen-day rat embryo. Proliferative zone. Note cell division. (Original magnification, ×1200; reduced ⅔.)

Proliferation and condensation of young fibroblasts continues in the area of the papilla. The future dentinoenamel junction shows no cusp outline. The accumulation of fibroblasts and capillaries around the body of the enamel organ and papilla is the dental follicle.

The aggregation of cells formed from both the ectoderm and the mesoderm may be called the tooth germ. It includes the enamel organ, the dental papilla, and the follicular wall. Bony encapsulation of the tooth germ has begun. The right and left palatine processes have joined. Some degenerating epithelium may be observed at the line of fusion (Fig. 10). In sagittal section, further anteroposterior growth of the dental lamina may be observed as well as the mesiodistal growth of the first molar tooth germ.

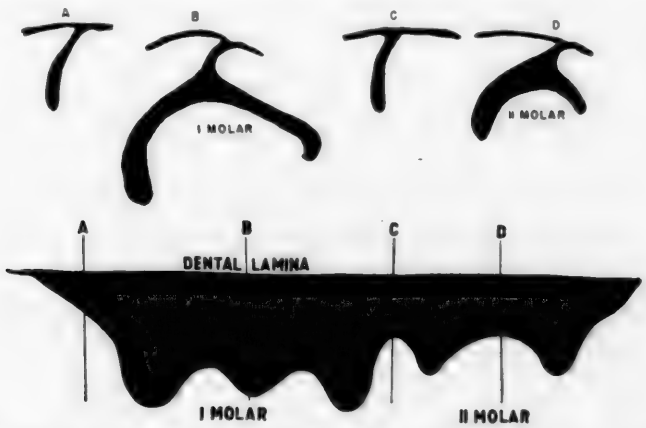


Diagram 2.—Eighteen-day rat embryo. To illustrate three-dimensional appearance of the dental lamina. *A, B, C,* and *D* on sagittal section represent corresponding frontal sections.

Eighteen Days.—The body of the enamel organ exhibits proliferative growth toward its predestined morphology. The proliferative zone has invaginated deeper into the mesenchyme, so that the body of the enamel organ resembles a cap (Fig. 13). When viewed serially the epithelial invagination exhibits the general configuration of the crown of the tooth. Posterior to this area it exists as a lamina in which all the peripheral cells are columnar, there being no proliferative zone. Further posteriorly, it widens again to form the second molar tooth germ which has progressed to the flask stage. Beyond the second molar tooth germ there is a slight extension of invaginating epithelial cells which will form the third molar (Diagram 2).

The oral mucosa shows a thin stratum corneum in isolated areas but not over the neck of the enamel organ. No change in the neck of the enamel organ is discernible.

Three groups of cells are still present in the body of the enamel organ in the same pattern of development. The central cells show increased vacuolization of the cytoplasm. The nuclei stain more intensely and are pyknotic. There

are numerous cystlike areas in which the cytoplasm is compressed. Small areas of cytoplasm are attached to the nuclei. These cells are undergoing senescence. The more recently formed central cells in the area of the proliferative zone show little senescence (Fig. 14). The cells of the middle layer are viable and change their morphology from round to squamous (Fig. 14). This layer is observed just inside the outer tunic and preameloblasts. The outer tunic has undergone further growth as a result of the invagination of the proliferative zone. There has been an increase in the area of the preameloblasts due to migration of the proliferative zone. No mitotic figures are seen in these cells (Fig. 14). The proliferative zone continues to migrate toward the cervix of the future tooth (Fig. 15).

The papilla exhibits growth and increased vascularity. The follicular wall shows no change. Bone growth in both the maxilla and mandible follows the pattern of the follicle in encapsulating the tooth germ. Fusion of the palatine process is complete. In sagittal section the anteroposterior growth of the lamina and tooth germ may be observed.

Nineteen Days.—Further growth of the body of the enamel organ has occurred by a deeper invagination of the proliferative zone, accentuating the cap stage (Fig. 16). It is now outlining the wall of the future dentinoenamel junction. The future dentin cusp is partially outlined.

The oral mucosa has a hornified layer which is continuous over the neck of the enamel organ (Fig. 17).

The central cells show an advanced stage of senescence. There is a marked reduction in the number of cells present. Many cells show minute areas of compressed cytoplasm or no cytoplasm at all. The nuclei are pyknotic. Microvacuoles have joined to form large vacuoles. The progress of the senescence of the central cells presents a picture of hydropic degeneration (Fig. 18). The cells of the middle layer appear viable and are found inside the outer tunic and adjacent to the preameloblasts (Fig. 18). Capillaries appear on the outer surface of the outer tunic, but do not extend to the dividing cells of the proliferative zone (Fig. 18). No vascularity exists within the body of the enamel organ. The cells of the newly formed outer tunic in the area of the proliferative zone are cuboidal. The preameloblasts from the time of their temporary differentiation have remained unchanged. They now show mitotic figures (Fig. 19). The increase in the number of cells occurs when the dentin cusp outline becomes evident. In the proliferative zone mitosis with ultimate differentiation continues to produce additional growth toward the cervix of the future tooth (Fig. 20).

The fibroblasts in the papilla have matured, exhibiting cell processes. They are denser in the area of the future dentinoenamel junction (Fig. 18). Encapsulation by the follicle has continued but does not sever the neck (Fig. 16). Anteroposterior growth of the lamina has its origin in the oral mucosa (Fig. 21). Mesiodistal growth of the first and second molar tooth germs is evident.

Twenty Days.—Growth of the enamel organ has occurred in the area of the cervix of the future tooth. Further development of the dentin cusp outline

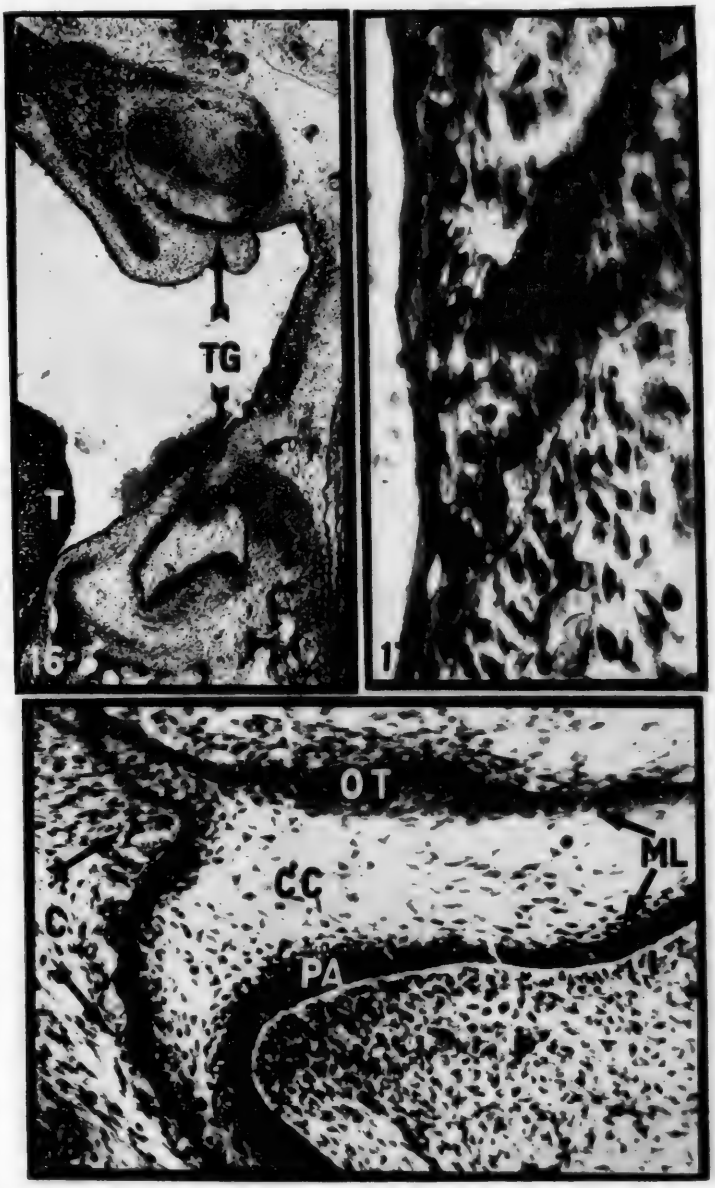


Fig. 16.—Nineteen-day rat embryo. Frontal section. *TG*, Tooth germ; *T*, tongue. (Original magnification, ×80; reduced ⅔.)

Fig. 17.—Nineteen-day rat embryo. Neck of mandibular tooth germ. Note hornified layer. (Original magnification, ×1200; reduced ⅔.)

Fig. 18.—Nineteen-day rat embryo. *CC*, Central cells; *ML*, middle layer; *OT*, outer tunic; *C*, capillaries; *PA*, preameloblasts; *P*, papilla. (Original magnification, ×400; reduced ⅔.)

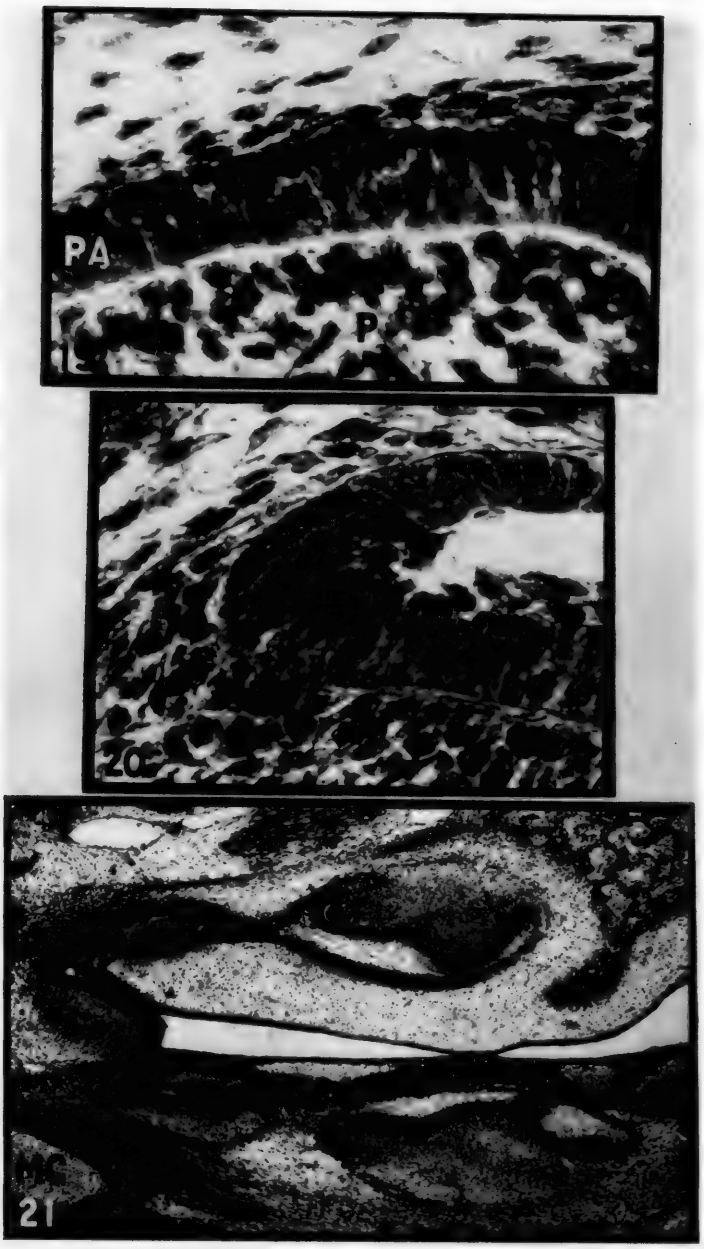


Fig. 19.—Nineteen-day rat embryo. *PA*, Preameloblasts, showing cell division; *P*, papilla. (Original magnification, ×1200; reduced ⅔.)

Fig. 20.—Nineteen-day rat embryo. Proliferative zone. Note cell division. (Original magnification, ×1200; reduced ⅔.)

Fig. 21.—Nineteen-day rat embryo. Sagittal section, showing anteroposterior growth. *MC*, Meckel's cartilage. (Original magnification, ×80; reduced ⅔.)

is evident (Fig. 22). The oral mucosa is now mature. An incomplete basal layer is present across the neck of the enamel organ and the stratum corneum is fully developed. Examination of sections stained by Wilder and Mallory methods shows a basement membrane underlying the oral mucosa, neck, outer tunic, and preameloblasts (Fig. 23). The area of the central cells shows a marked change. The highly vascular follicle has penetrated the outer tunic bringing capillaries and fibroblasts into the central cell area (Fig. 24). The outer tunic has dedifferentiated into round epithelium which now is confluent with the adjacent middle layer (Fig. 24). In the area of the preameloblasts the middle cells present a layer of squamous epithelium about three cells thick. This is the stratum intermedium (Fig. 25). The mitosis of the preameloblasts continues, and appears to be associated with cusp formation (Fig. 25). The proliferative zone continues in the pattern of that observed at 19 days (Fig. 26).

Vascularity in the papilla has increased. Partial encapsulation of the tooth germ by the follicle and bone continues (Fig. 22).

Further anteroposterior growth of lamina may be observed in sagittal section (Fig. 27). Between the first and second tooth germs the lamina stage, which is continuous with the oral mucosa, may still be observed. Posterior to the second molar tooth germ the lamina which will form the third molar is evident. It originates from the basal layer of the oral mucosa.

Twenty-one Days.—The tooth germ has undergone additional growth in the area of the cervix of the future tooth. The outline of the dentinoenamel junction is almost complete, and exhibits cusp outline in these areas (Fig. 28).

The oral mucosa has the appearance of adult tissue and is continuous over the neck of the enamel organ. An incomplete basal layer extends across the neck (Fig. 29).

There is an increase of fibroblasts and capillaries in the area previously occupied by the central cells. The outer tunic is no longer a continuous band of epithelium, but is broken up into islands of chromophobic cells by the invading follicular wall (Fig. 29). The tooth germ now consists of the follicular wall encapsulating the stratum intermedium, preameloblasts, proliferative zone, and papilla. The stratum intermedium is present adjacent to the preameloblasts (Fig. 30). The preameloblasts continue to divide (Fig. 30). The proliferating zone continues to divide and invaginate, producing additional growth (Fig. 31). A basement membrane separates the preameloblasts from the papilla, which is a dense mass of undifferentiated fibroblasts. Vascularity in the area has increased (Fig. 29).

Bony encapsulation has progressed from the apical area, and appears to be surrounding the tooth germ except in the occlusal portion (Fig. 28). In sagittal section the anteroposterior growth of the lamina and growth of the first molar tooth germ may also be observed.

Birth.—The general appearance of the tooth germ resembles the crown of the tooth. The additional growth has occurred in the area of the proliferative zone, which at this time presents the constriction of the cervix of the tooth. The outline of the dentin cusp has developed further (Fig. 32).

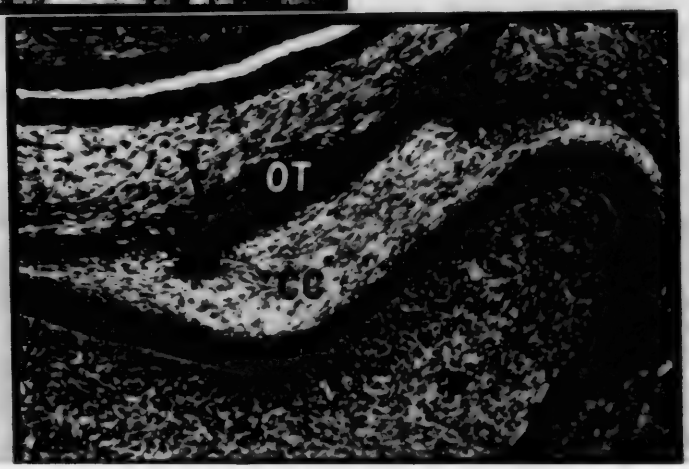


Fig. 22.—Twenty-day rat embryo. Frontal section. *TG*, Tooth germ; *T*, tongue. (Original magnification, ×47; reduced ⅔.)

Fig. 23.—Twenty-day rat embryo. Wilder stain. Arrows point to basement membranes underlying oral mucosa, outer tunic, and preameloblasts. (Original magnification, ×250; reduced ⅔.)

Fig. 24.—Twenty-day rat embryo. *CC*, Central cells; *OT*, outer tunic. (Note capillaries at outer tunic. Arrow points to follicular wall invasion of central cell area.) *P*, Papilla. (Original magnification, ×250; reduced ⅔.)

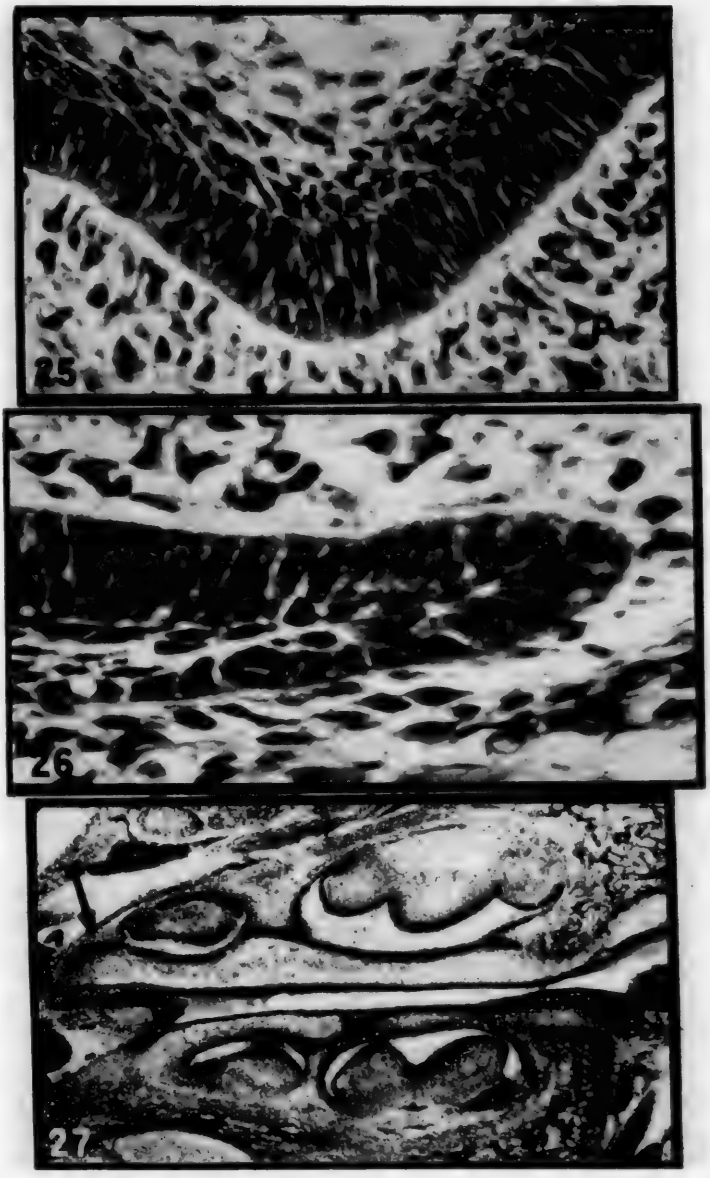


Fig. 25.—Twenty-day rat embryo. *SI*, Stratum intermedium; *PA*, preameloblasts, showing cell division; *P*, papilla. (Original magnification, ×250; reduced ⅔.)

Fig. 26.—Twenty-day rat embryo. Proliferative zone. Note cell division. (Original magnification, ×1200; reduced ⅔.)

Fig. 27.—Twenty-day rat embryo. Sagittal section. Note development of first and second molar tooth germs. Arrow points to distal extension of lamina which will form third molar. (Original magnification, ×27; reduced ⅔.)

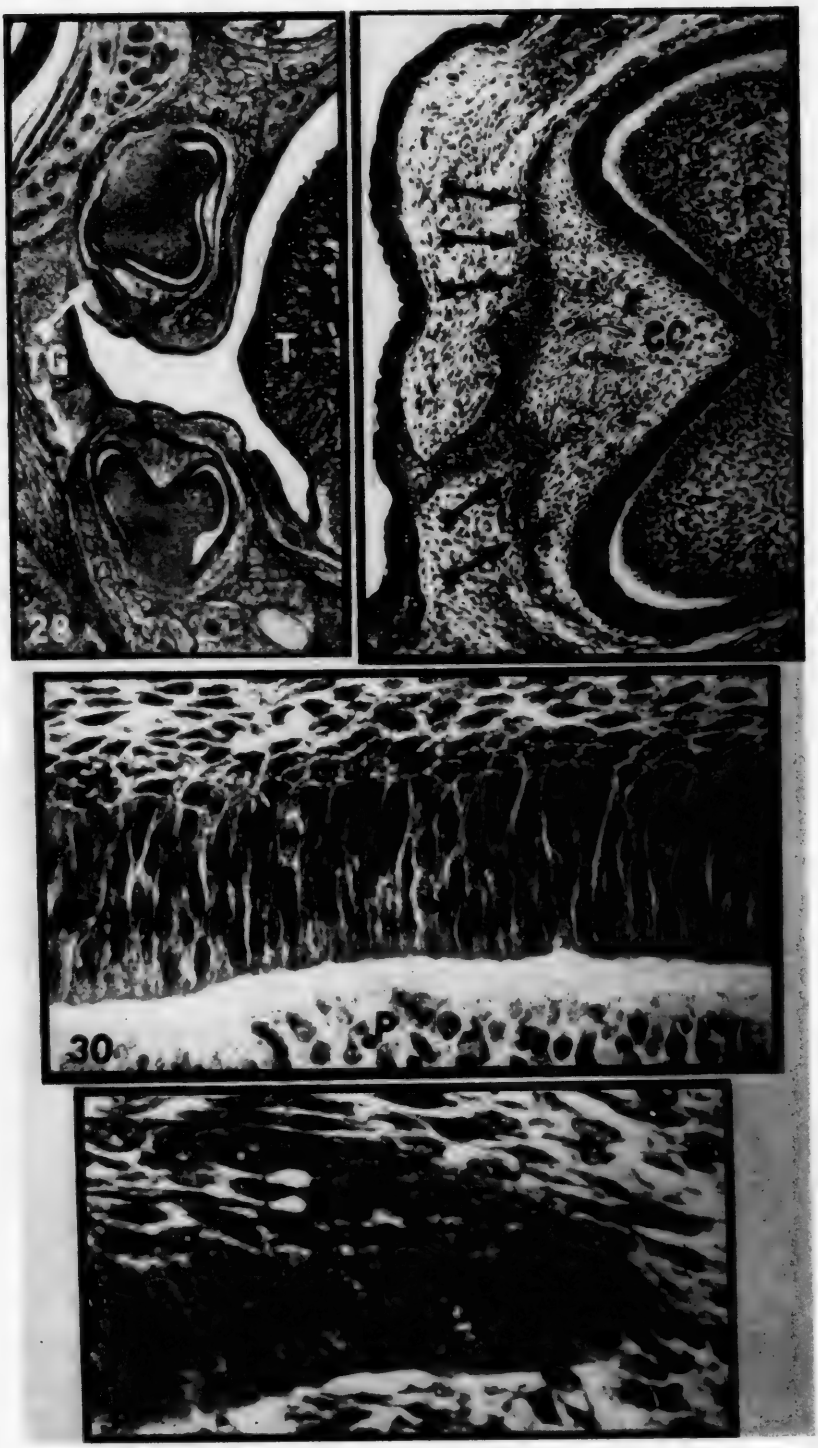


Fig. 28.—Twenty-one-day rat embryo. Frontal section. *TG*, Tooth germ; *T*, tongue. (Original magnification, ×32; reduced ⅔.)

Fig. 29.—Twenty-one-day rat embryo. *CC*, Central cells. (Arrows point to follicular wall invasion of outer tunic.) *P*, Papilla. Note mature oral mucosa. (Original magnification, ×200; reduced ⅔.)

Fig. 30.—Twenty-one-day rat embryo. *PA*, Preameloblasts (note cell division); *SI*, stratum intermedium; *P*, papilla. (Original magnification, ×1200; reduced ⅔.)

Fig. 31.—Twenty-one-day rat embryo. Proliferative zone. Note cell division.

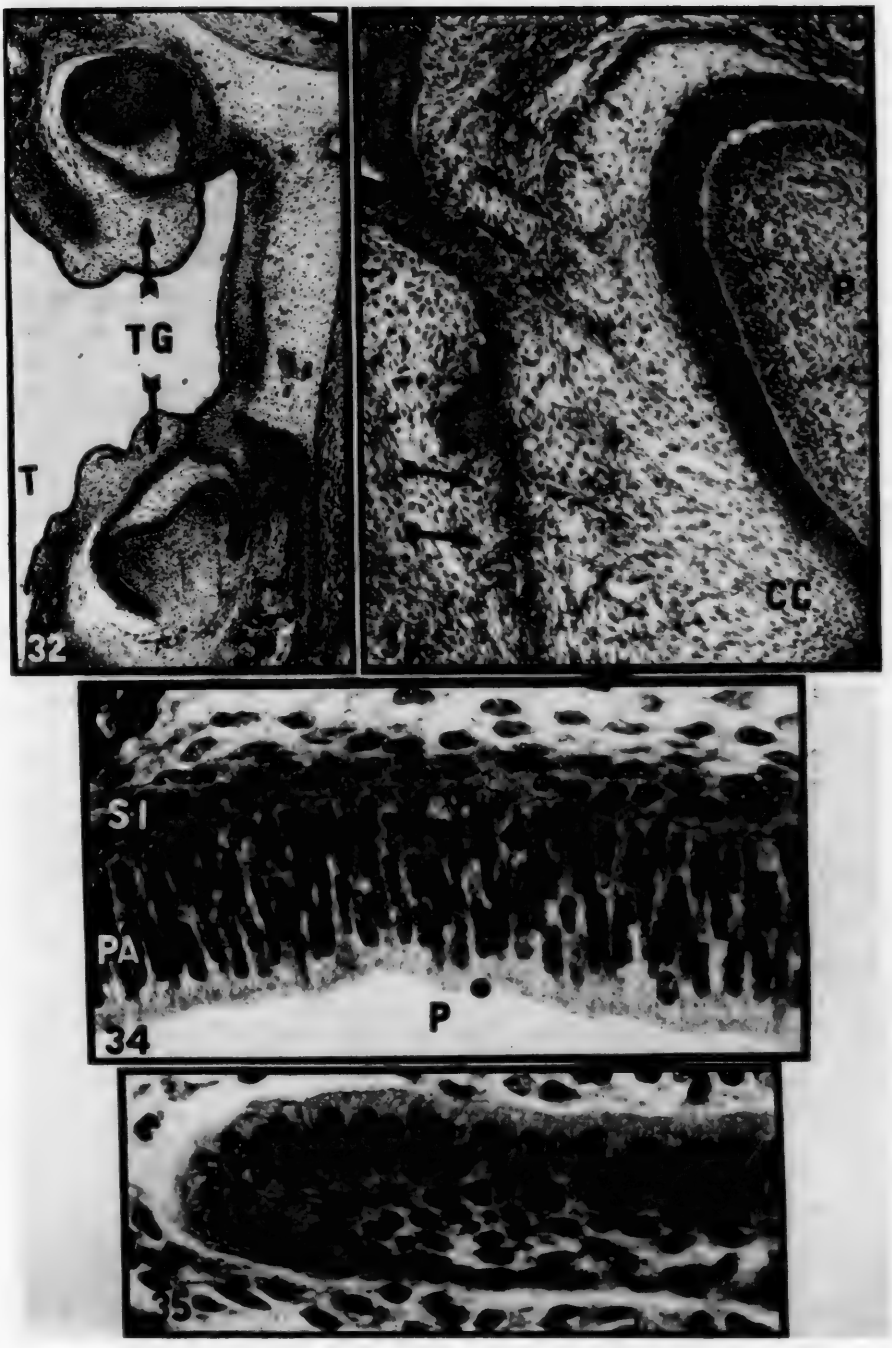


Fig. 32.—At birth. Frontal section. *TG*, Tooth germ; *T*, area of tongue. (Original magnification, ×85; reduced ⅔.)

Fig. 33.—At birth. *CC*, Area of central cells. (Arrows point to follicular wall invasion of outer tunic. Note fibrous tissue in area of central cells.) *P*, Papilla. (Original magnification, ×250; reduced ⅔.)

Fig. 34.—At birth. *SI*, Stratum intermedium. (Note morphologic change.) *PA*, preameloblasts, showing cell division; *P*, area of papilla. (Original magnification, ×1200; reduced ⅔.)

Fig. 35.—At birth. Proliferative zone. Note cell division. (Original magnification, ×1200; reduced ⅔.)

The neck of the enamel organ and the oral mucosa remain the same as that seen at 21 days of embryonic development. The connective tissue and vascular invasion through the outer tunic has progressed (Fig. 33). It extends up to, but does not penetrate, the proliferative zone. The area of the central cells has increased in vascularity and exhibits more mature fibrous tissue (Fig. 33). In the occlusal area the inner layer of the stratum intermedium has begun to differentiate (Fig. 34). These cells are no longer squamous, but begin to assume their future cuboidal form, except over the tips of the cusps where they remain unchanged. The preameloblasts continue to divide as cusp formation advances (Fig. 34). Growth has continued in the area of the proliferative zone (Fig. 35). Mitosis is active and the proliferative zone still gives rise to cells as it forms the outline of the cervix.

Fibroblasts at the periphery of the papilla have increased in density and differentiation of odontoblasts has begun (Fig. 37). The basement membrane has increased in thickness and can now be identified as dentin (Fig. 36). Vascularity of the papilla has increased. The follicular wall and bony encapsulation have enveloped the tooth germ up to the neck of the enamel organ (Fig. 32).

In sagittal section, the dentin cusp is completely outlined. The lamina joining the first and second molars is present. A distal extension of the lamina to form the third molar may be observed posterior to the second molar tooth germ (Fig. 38).

DISCUSSION

A detailed description of the daily changes in odontogenesis of the rat molar has been made from 13 days embryonic age to birth. The discussion will present the dynamics of odontogenesis, as well as the cytomorphosis of each element and its function.

Proliferative Zone.—Odontogenesis is initiated by a group of cells which originate in the basal layer of the oral mucosa of the future ridges. These are the cells of the proliferative zone which divide and invaginate the mesenchyme, leaving behind a band of undifferentiated cells bordered by columnar epithelium. At 16 days these cells reach their optimum depth and the proliferative zone changes its position to the walls of the fundus. Further growth occurs peripherally corresponding to the width of the coronal dentinoenamel junction. The proliferative zone then invaginates further to outline the walls of the crown and roots of the tooth (Figs. 3, 6, 8, 12, 15, 20, 26, 31, and 35).

Growth is a function of the proliferative zone. It outlines the periphery of the dentin from the crown to the apices of the roots and also gives rise to the cells of the outer tunic, preameloblasts, central cells, and stratum intermedium.

Outer Enamel Epithelium or Outer Tunic.—The epithelium located at the periphery of the body of the enamel organ is known as the outer tunic or outer enamel epithelium (Diagram 1). The designation "outer enamel epithelium" implies a contribution to amelogenesis. A progressive study of the cytomorphosis of these cells establishes that they are not concerned with the formation of enamel.[15] The older term, "outer tunic," is a more appropriate designation.

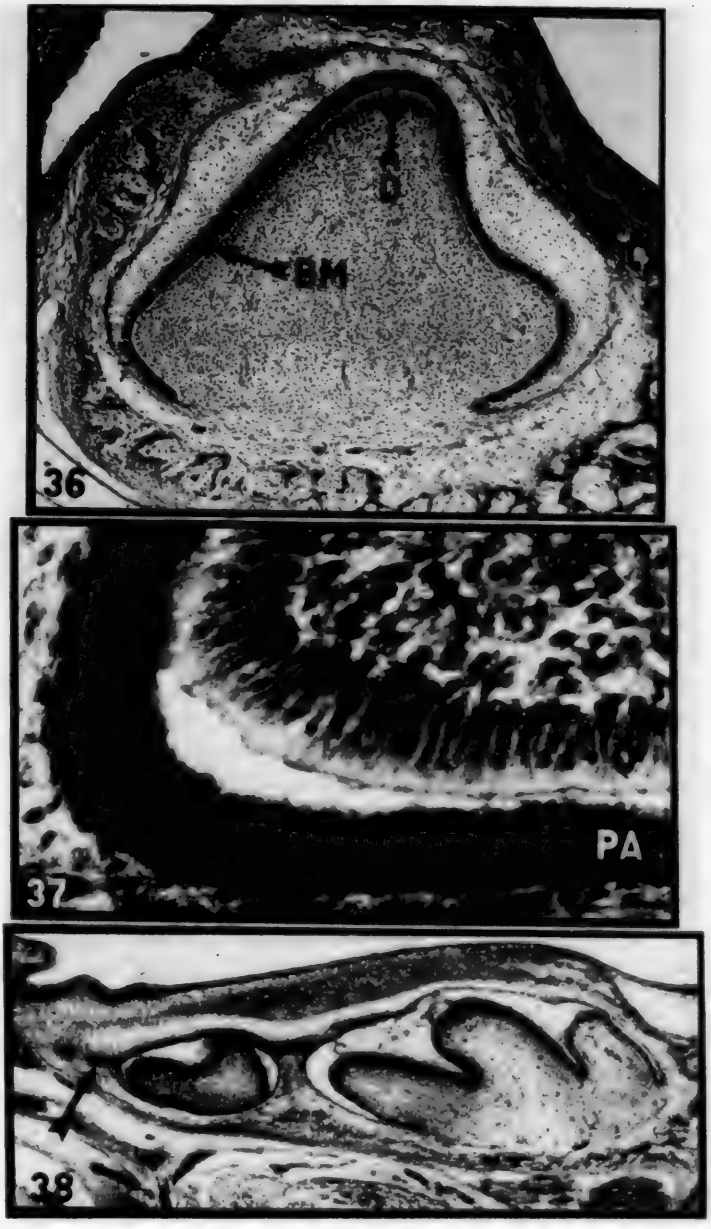


Fig. 36.—At birth. (Wilder stain.) *D*, Dentin, *BM*, basement membrane. Note reticular fibers in area of central cells and bony encapsulation. (Original magnification, ×120; reduced ⅔.)

Fig. 37.—At birth. *O*, Odontoblasts; *PA*, preameloblasts. (Original magnification, ×910; reduced ⅔.)

Fig. 38.—At birth. Sagittal section. Arrow points to lamina to form third molar.

The outer tunic is a band of columnar epithelium continuously left behind as the proliferative zone invaginates. From the time of differentiation to the twentieth day of embryonic development it shows no apparent morphologic change. Capillaries appear at the outer border of the outer tunic at 19 days. Twenty-four hours later, the columnar epithelium of the outer tunic becomes squamous and the more numerous capillaries now extend from the neck of the enamel organ to the proliferative zone. Capillaries and fibroblasts of the follicular wall penetrate the outer tunic, leaving a residue of epithelial rests. At 21 days, the neck and the epithelial rests of the outer tunic are separated from the remainder of the enamel organ by a young follicular wall (Figs. 11, 14, 18, 24, 29, and 33). The tooth germ now consists of the stratum intermedium, preameloblasts, proliferative zone, and papilla encapsulated by the follicular wall.

The function of the outer tunic is to provide a band of epithelium from the oral mucosa to the proliferative zone, similar to that found in the formation of the endocrine glands. Having achieved this function, the cells of the outer tunic are subsequently absorbed.

Stratum Intermedium or Middle Layer.—The stratum intermedium has been described as a layer of squamous cells just exterior to the preameloblasts and is thought to be essential to the development of enamel, and may contribute to the process of calcification.[16, 17]

At 17 days the middle layer of cells appears as round epithelium, four to five cells thick, extending around the entire body of the enamel organ. At 18 days the cells become squamous. At 20 days, when the outer tunic dedifferentiates into round epithelium, it fuses with the middle layer in this area, and the two layers cannot be distinguished from each other (Figs. 11, 14, 18, and 24).

In the area of the preameloblasts the middle layer develops into the stratum intermedium. At 18 days the cells change from round to squamous. At birth the layer of cells immediately adjacent to the preameloblasts begins to change from squamous to its eventual cuboidal form. Over the tips of the cusps where no enamel will form this change is not evident (Figs. 14, 30, and 34). The function of the cells of the stratum intermedium may be to exert an organizer influence on the preameloblasts.

Stellate Reticulum or Central Cells.—The area of the central cells has been named the stellate reticulum. It has been described as an epithelial structure containing a large quantity of fluid and consisting of stellate epithelial cells joined together by long intercellular bridges.[18] C. F. W. Bodecker[19] described it as a myxomatous tissue, and assumed that a metaplasia of the original epithelium occurred. Noyes, Schour, and Noyes[20] described it as a cellular network which resembled embryonic connective tissue or mesenchyme of epithelial origin.

The central cells are short-lived. From 14 to 16 days they appear similar to the superficial cells of the oral mucosa and are continuous with them. At 17 days the first stage of senescence may be observed in the midmost area. Minute vacuoles appear in the cytoplasm. During the eighteenth and nineteenth days the process of degeneration continues. The cytoplasm, replaced by

larger and larger vacuoles, appears as strands similar to that seen in hydropic degeneration.[21] The cells are fewer in number; the nuclei are irregular in form and pyknotic. Only minute areas of cytoplasm are attached to the nuclei. The area becomes cell poor. Long strands of compressed cytoplasm connect the remaining pyknotic nuclei. This may be described as the senescent stage of the central cells of the enamel organ (Figs. 11, 14, and 18).

Prior to 20 days, the enamel organ is an avascular structure. At 19 days capillaries appear outside the outer tunic.[15] At 20 days capillaries and fibroblasts from the follicle penetrate the outer tunic and invade the central area. At birth a loose areolar fibrous tissue may be observed in the central area of the enamel organ (Figs. 14, 18, 24, 29, and 33).

Replacement of the central cells by fibrous tissue of the follicular wall establishes a free internal organ. In the monophyodont dentition of the rat the mesenchyme invasion does not develop a successional lamina and enamel niche such as is found in a diphyodont dentition.[28]

The function of the central cells is to occupy the lamina and enamel organ. This view was also expressed by Addison and Appleton.[15] It is unlikely that the central cells could perform functions attributed to them,[16, 17] particularly since they are replaced by connective tissue from the follicular wall before amelogenesis begins.

The presence of a blood supply in the area formerly occupied by the original epithelial cells has been associated with the need for metabolites in the formation of enamel.[18] This area, now occupied by a young follicular wall, exhibits a normal blood supply. The vascularity may be related to amelogenesis, but methods other than histologic must be utilized to establish the connection.

The condensation of epithelial cells in the central area of the body of the enamel organ known as the enamel knot or cord[22] is an artifact caused by the oblique cutting of the tooth germ or a cut oblique to the preameloblasts (Figs. 10, 13, and 21). Even in a perfect orientation there are sections where the preameloblasts are cut obliquely. When they are so cut, the middle layer of viable cells appears more than the usual four to five cells in thickness. Examination of serial sections demonstrates that the more oblique the cut, the more definite is the condensation of epithelial cells designated the enamel knot or cord.

Preameloblasts.—The preameloblasts, arising from the proliferative zone, have differentiated into columnar epithelium at 17 days. They are longer than the columnar epithelium of the outer tunic. The nuclei are large and centrally located. The differentiation of the preameloblasts is temporary. At 19 days the presence of mitotic figures in the preameloblast layer indicates modulation.[23] These cells, with the exception of those over the tips of the dentin cusps, will differentiate again postnatally into ameloblasts (Figs. 19, 25, 30, and 34).

There is a relation between the morphogenesis of the dentin cusp outline and mitosis of the preameloblasts. Prior to 19 days the preameloblasts show no mitosis and present a smooth dentinoenamel junction. With the appearance of mitotic figures in the preameloblast layer, the first development of cusp outline is also observed.

Cusp outline, which occurs after formation of the walls of the crown, would require either a widening of the cells or cell division to accommodate the increased linear dimensions of the dentinoenamel junction. The profusion of mitotic figures observed in the preameloblast layer from 20 days embryonic growth to birth establishes that the cusp outline at the dentinoenamel junction is caused by cell division. The mitotic activity is greatest in the areas of the dentin groove and tips of the cusps.

Neck.—In the early stages no changes are evident in the neck of the enamel organ. At 19 days a thin stratum corneum appears. At 20 days the oral mucosa becomes mature and has basal, prickle cell, and granular layers, and a thick stratum corneum. The neck of the enamel organ is still present eight days after birth (Figs. 17, 22, 29, and 32). The neck is the first-formed part of the dental lamina and functions as part of the oral mucosa.

Papilla.—The papilla is established when the concavity first appears in the preameloblast layer. As the enamel organ grows the papilla increases in size. The condensation of cells in the papilla is the result of cell division of the fibroblasts. The condensation of fibroblasts is greater and the cells are larger in the area of the dentinoenamel junction. There is a constant increase in vascularity in the deeper portion of the papilla (Figs. 10, 13, 18, and 21).

At 19 days the fibroblasts mature and exhibit cell processes. Collagenous fibers appear in the intercellular substance throughout the papilla, but at the dentinoenamel junction they arrange themselves into a basement membrane. The basement membrane increases in thickness up to birth, when it can be identified as predentin (Figs. 19, 23, 29, and 37). When the fibroblasts differentiate into odontoblasts, they withdraw from the dentinoenamel junction. Processes of the fibroblasts already embedded in the basement membrane remain in situ. As the cells withdraw the processes join to form Tomes's fiber. The embedded processes are the terminal branches of the dentinal tubules.

Differentiation of odontoblasts is first observed at birth. It is preceded by the formation of the first layer of predentin, the basement membrane. There is no calcified dentin at birth.

Follicular Wall.—The formation of the follicular wall is first observed at 17 days. It is a loose arrangement of fibroblasts and capillaries enveloping the papilla and enamel organ but does not sever the neck. At 19 days it appears as a fibrous capsule. At 20 days it penetrates the outer tunic, and at birth a young fibrous follicular wall develops in the area of the central cells of the enamel organ (Figs. 10, 16, 23, 24, 29, 33, and 36).

Bony encapsulation begins at 18 days. It follows the pattern of the follicular wall, beginning at the base of the tooth germ, and proceeds toward the neck.

Organizer Influence.—The organizer influence or mutual action of one group of cells upon another is a fundamental law of organogenesis and histodifferentiation. Organizers in odontogenesis have been discussed by many investigators.[8, 9, 24, 25]

Histologic study provides no answer to the question of the organizer which causes the dental lamina to form. In appearance it is similar to other bands which arise as epithelial invaginations. The condensation of fibroblasts occurs simultaneously. A suggestion that the mesenchyme stimulates the invagination of epithelium has recently been made.[26] Most investigators subscribe to the theory that the epithelium initiates the activity in the mesenchyme.[22]

It is accepted that ameloblasts influence the differentiation of odontoblasts, and that dentin is essential to enamel formation.[21] This report describes a preameloblast stage which precedes the ultimate differentiation of ameloblasts. At birth the preameloblasts are undergoing cell division prior to becoming ameloblasts. It is during the time of active mitosis of the preameloblasts that differentiation of odontoblasts occurs (Figs. 34 and 37).

Differentiation of the ameloblasts occurs after dentin formation begins. Over the enamel-free cusps the preameloblasts do not differentiate into ameloblasts. Differentiation of odontoblasts, however, has occurred in this area, suggesting that the organizer influence exists in the preameloblast stage (Fig. 36). The concept that the presence of dentin stimulates enamel formation is questionable because dentin is present in the area of the cusp tip and amelogenesis does not occur here.

Histodifferentiation of the ameloblasts is preceded by a morphologic change in the cells of the stratum intermedium adjacent to the preameloblasts (Fig. 34). They become cuboidal with basally located nuclei, except in the area of the enamel-free cusp tip. Here they remain as squamous epithelium, even though dentin formation has begun. It appears likely that the stratum intermedium exerts an organizer influence on the preameloblasts rather than on the dentin.

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THE PERIODONTIUM AND SALIVARY GLANDS IN THE ALARM REACTION

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THE development by Selye of the concept of the general adaptation syndrome and the diseases of adaptation[1] introduced a new viewpoint regarding the nature of disease processes. According to this concept, the reaction of the body to a wide variety of damaging influences or stress-producing agents such as trauma, severe cold, muscular fatigue, drug intoxications, and nervous stimuli[2] consists of a composite of nervous and endocrinologic alterations which in turn produce a syndrome of somatic changes. Prominent among the latter changes are enlargement of the adrenal cortex, involution of lymphatic tissue such as thymus and spleen, and gastrointestinal ulceration.[3-5] The sum of these nonspecific systemic reactions is termed the "general adaptation syndrome," which consists of the following three more or less distinct phases,[6] depending upon the duration of exposure and the severity of the stimuli:

The alarm reaction is the sum of all nonspecific phenomena elicited by sudden exposure to stimuli to which the organism is insufficiently adapted. Some of these reactions are due to damage or shock, while others represent defensive mechanisms against the damage. Increased activity of the adrenal cortex with enlargement of the gland is characteristically seen in the alarm reaction.

The resistance stage represents the sum of all nonspecific systemic reactions elicited by prolonged exposure to stimuli to which the organism has acquired adaptation.

The exhaustion stage is the sum of all nonspecific systemic reactions which eventually occur due to prolonged exposure to stimuli to which adaptation was acquired but could not be maintained.

It has been noted that osteoporosis[7-10] can develop during the general adaptation syndrome, particularly in the alarm reaction and the exhaustion stage. The finding of osteoporosis in the general adaptation syndrome is most interesting from the periodontal viewpoint. It suggested the possibility that if generalized osteoporosis were induced by stress, the latter might be a factor in causation of alveolar bone loss in chronic destructive periodontal disease.[11-13]

There have been limited clinical or experimental studies which have dealt with the problem of the effect of stress upon the periodontal tissues. There are studies which may have some pertinence to the subject. Since the changes of the general adaptation syndrome are mediated by the adrenocorticotropic

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hormone of the pituitary gland and the various adrenocortical hormones,[6] experiments which deal with the effects of these substances upon tissues of the periodontium or bone in general are of interest. Glickman, Stone, and Chawla[14] noted osteoporosis of alveolar bone, and degeneration and reduction in number of the collagen fibers of the periodontal membrane, in mice subjected to repeated injections with cortisone. Reduced osteoblastic activity in the skeletal system in areas other than alveolar bone has been reported following injections with adrenocorticotropic hormone and cortisone.[15-18]

Based partly upon the suggestive evidence of these reports, and in view of the absence of more specific information, the need was felt for exploring the effects of stress upon the tissues of the periodontium. A series of experiments was undertaken for this purpose. The findings in the alarm reaction are reported here.

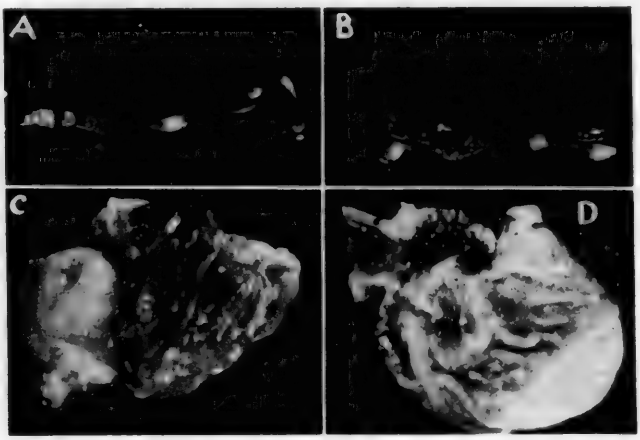

Fig. 1.—*A*, Spleen in control animal; *B*, spleen in the alarm reaction showing marked decrease in size; *C*, gastric mucosa in control animal; *D* gastric mucosa in the alarm reaction showing roughened surface and ulceration.

EXPERIMENTAL PROCEDURE

Fifteen female albino rats, Wistar strain, 2½ to 3 months of age, weighing 190 to 210 Gm., were used as experimental animals. Female animals were used because of a tendency toward a more severe reaction to stress.[5] Subcutaneous injections of 4 per cent formaldehyde were selected as a simple method of inducing stress. Subcutaneous injection of formaldehyde leads to local coagulation-necrosis of tissue. With local injection of small doses, the systemic effects are not severe and no notable pathologic changes are seen in the major organs. The drug has been commonly used in the experimental production of the alarm reaction.[6]

The animals were divided into control and experimental groups. The experimental group of ten animals received four injections of 4 per cent formaldehyde within forty-eight hours. Each injection, consisting of 0.5 c.c. of

the solution, was administered subcutaneously in the groin. Food was withheld during the forty-eight-hour course of the experiment so as to enhance the stress reaction, but water was given ad libitum. Five litter mates served as controls.

At the termination of the experimental period the animals were sacrificed with ether. The adrenals, thymus, spleen, salivary glands, heart, liver, kidney, lung, stomach, and cervical lymph nodes were removed and fixed in 10 per cent formalin solution for microscopic study. The mandible and femur were fixed in formalin and decalcified by the formic acid-sodium citrate technic.[19] Tissues were sectioned at 7 microns, and stained with hematoxylin-eosin and Mallory connective tissue stains.

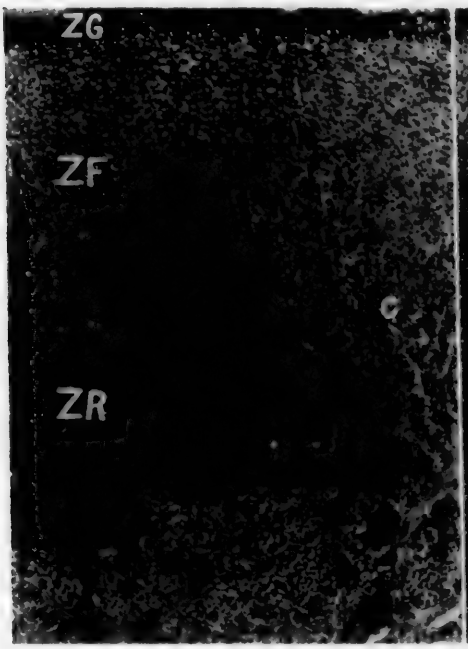

Fig. 2.

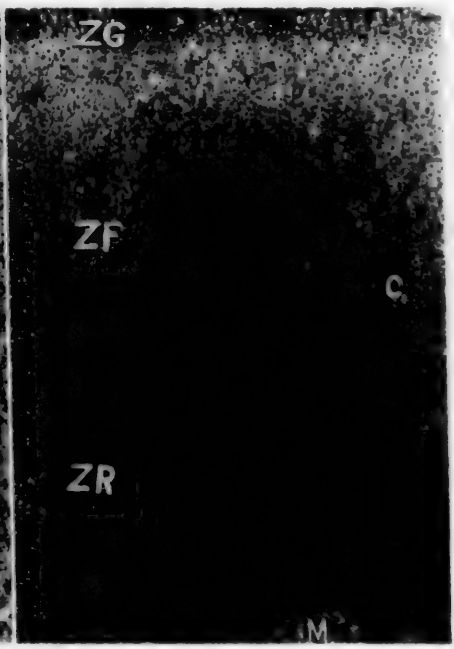

Fig. 3.

Fig. 2.—Section of adrenal gland in control animal. The adrenal cortex *C* is sharply demarcated from the adrenal medulla *M*. The cortex (note normal width) is clearly separated into zona glomerulosa *ZG*, zona fasciculata *ZF*, and zona reticularis *ZR*. Spaces occupied by lipid granules are seen in the zona fasciculata. Zone glomerulosa is particularly well demarcated from the zona fasciculata. (Hematoxylin-eosin stain. Original magnification, ×160, reduced 3/7.)

Fig. 3.—Section of adrenal gland in the alarm reaction. The width of the cortex *C* is considerably increased. Zona glomerulosa *ZG* blends into zona fasciculata *ZF*. The zona reticularis *ZR* is not distinct from zona fasciculata. Lipid spaces are not present in zona fasciculata, cells are hypertrophic, and "cord" appearance is indistinct. Areas of cytolysis are apparent. Entire cortex is hyperemic. Medulla *M* appears normal. (Hematoxylin-eosin stain. Original magnification, ×160, reduced 3/7.)

OBSERVATIONS

In all the experimental animals the site of injection was ulcerated and microscopically presented coagulation necrosis of the connective tissue and muscle. Generalized subcutaneous edema was seen in several of the experi-

mental animals. The adrenal glands were enlarged and darker in color than those of the control animals. The thymus gland was reduced in size and appeared pale and soft. The spleen was decreased in size and the gastric mucosa in several animals showed areas of deep ulceration with hemorrhage (Fig. 1).

Microscopically, the experimental animals presented the following changes.

Adrenal Gland.—The adrenal cortex was increased in width (Figs. 2 and 3). The demarcation between the zona glomerulosa and zona fasciculata was poorly defined. The cells in the latter zone appeared devoid of their lipid granules. Nuclei in the zona glomerulosa were less densely arranged due to cellular hypertrophy. The normal parallel cordlike arrangement of the cells in the zona fasciculata was not apparent. There was evidence of cytolysis, and hyperemia was a notable feature.

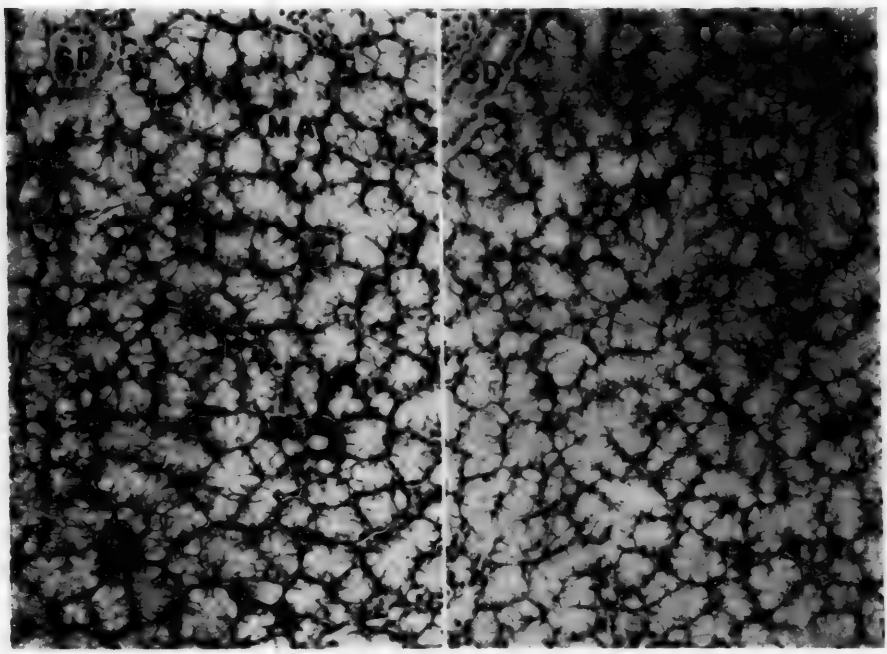

Fig. 4. Fig. 5.

Fig. 4.—Section of sublingual gland in control animal. The mucous acini are regular and well formed. There is a concentration of basophilic material at the base of secretory cells, and granules are apparent in the clear cytoplasm. *MA*, Mucous acini; *SD*, secretory ducts. (Hematoxylin-eosin stain. Original magnification, ×250, reduced ⅔.)

Fig. 5.—Section of sublingual gland in the alarm reaction. There is a reduction in the amount of basophilic material at the base of secretory cells. The cytoplasmic granules are not apparent. Some disruption of normal acinar architecture is seen. *MA*, Mucous acini; *SD*, secretory ducts. (Hematoxylin-eosin stain. Original magnification, ×250, reduced ⅔.)

Thymus.—The cortical areas of the thymus showed degenerative changes. The thymocytes were less densely arranged than normal and showed evidence of pyknosis. The connective tissue reticulum appeared prominent due to pyknosis of the cortical cells.

Salivary Glands.—The sublingual and submaxillary glands presented comparable changes. The normal architecture was disrupted in scattered areas (Figs. 4 and 5). The basophilic material and cytoplasmic granules of the parenchymal cells were markedly diminished. Elements of the connective tissue stroma were reduced in amount so that the acini appeared very thin-walled and delicate.

Periodontium.—No notable microscopic changes were seen in the gingiva, periodontal membrane, alveolar bone, or cementum.

Pulp.—The pulp of the molar teeth of several experimental animals showed degenerative changes in the odontoblastic layer which were not observed in the control animals. These consisted of nuclear pyknosis and disruption of the regular linear arrangement of the odontoblasts.

DISCUSSION

Interpretation of the findings is subject to the qualifying limitations imposed by the small group of animals and the fact that only one type of stressor agent was studied. For these reasons, this study must be considered as an exploratory one.

The findings indicate that the alarm reaction was produced in the experimental animals by the subcutaneous injections of formaldehyde. The gross and microscopic changes seen in the various organs such as the adrenal glands, thymus, spleen, and gastrointestinal tract were consistent with those reported as being characteristic of the alarm reaction.[6, 20, 21] The regressive changes in lymphatic organs such as the thymus, spleen, and cervical lymph nodes were not as severe as those described in the literature. The absence of notable changes in the periodontium was in marked contrast to the alterations observed in the aforementioned tissues. It very well may be that the periodontal tissues were altered in this experiment, but that the alterations were of such a degree and nature that they could not be detected by the hematoxylin-eosin or Mallory stains. It is particularly noteworthy that there was no evidence of osteoporosis in the long bones or in the alveolar bone.

It may be that the forty-eight-hour period of the alarm reaction was insufficient to produce changes in the periodontium. The possibility that changes in the periodontal tissues may occur in animals subjected to stress for a sufficient period of time to lead to the exhaustion stage of the general adaptation syndrome, is not ruled out by our findings. However, the nature of the stressor mechanism employed in this experiment precluded an experimental period of sufficient duration to produce the exhaustion stage.

The degenerative changes seen in the salivary glands are noteworthy and have been reported before.[22] Their significance remains to be explored.

SUMMARY

The alarm reaction of the general adaptation syndrome was produced in albino rats by using repeated injections of 4 per cent formaldehyde as the stressor mechanism. The presence of the alarm reaction was demonstrated by

gross and microscopic changes in the adrenal glands, thymus, spleen, and gastrointestinal tract. No notable gross or microscopic changes were observed in the periodontal tissues. Degenerative changes in the salivary glands were noted in the alarm reaction.

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WATER-SOLUBLE MUCOPROTEINS OF THE GINGIVA

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THE morphologic changes of the gingiva in inflammation and in certain pathologic states are accompanied by chemical differences which have been partly defined by histochemical means.[1-3] For example, in desquamative gingivitis the extracellular glycoprotein ground substance of the connective tissue[1] contains increased amounts of water-soluble, alcohol-insoluble carbohydrate.

The ground substance of the connective tissues is conceived to exist largely as a highly aggregated structure which is highly plastic in a biologic sense. Thus its chemical and physical behavior is readily modified in physiologic and pathologic states.[4, 5] The lability of ground substance may account in part for the response of the gingiva to local injury and to changes in the internal milieu. The extent of some of these connective tissue changes in gingival tissue has now been quantitatively evaluated. The soluble mucoproteins of the tissue were extracted, and determination by analytic procedures showed them to be increased in gingival inflammation.

MATERIAL AND METHOD

Gingival tissue was obtained by biopsy from patients who were having teeth extracted or receiving periodontal treatment.* The state of the tissue was remarked, and it was classified as normal or inflamed. Such clinical criteria as color, swelling and edema, and bleeding were used in estimating the degree of inflammation. However, the main purpose was to differentiate healthy from diseased tissue. The biopsy specimens, consisting of marginal and attached gingiva involving several papillae, were removed following block anesthesia or infiltration into an adjacent area. These specimens were immediately frozen in liquid nitrogen at about –190° C. and then prepared by freezing-drying in vacuo at approximately –30° C. A small piece of the frozen-dried preparation was reserved for histochemical examination. The remainder was used for quantitative determinations of water-soluble mucoprotein. A total of fifty-three specimens comprised of fifteen from healthy gingiva and thirty-eight from inflamed gingiva were evaluated.

Determination of Water-Soluble Mucoprotein.—Approximately 30 mg. of the dried tissue was finely divided and defatted in acetone at –2° to –5° for three hours. The acetone was decanted following a short centrifugalization in a chilled rotor, and residual acetone was removed by suction. These steps were

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carried out rapidly at low temperature to minimize possible denaturation of the mucoproteins. The residue was brought to constant weight, and then ground with exactly 2.0 ml. of M/15 phosphate buffer (pH 7), and the soluble material was extracted for one hour in the cold. A measured portion of this extract was withdrawn, and the mucoprotein was determined by the method of Winzler, Devor, Mehl, and Smyth.[6] Briefly, this involves removal of other proteins with perchloric acid and then precipitation of the mucoproteins from the filtrate with phosphotungstic acid. The mucoproteins are determined as carbohydrate and as tyrosine.

Histochemical Methods.—The frozen-dried tissues were infiltrated with paraffin (melting point 60° to 62° C.), and sections were cut at 4 to 8 microns. After being deparaffinized in xylol or petroleum ether, the tissues were denatured overnight in 95 per cent ethanol, and then stained for carbohydrate-containing substances with the periodic acid-leucofuchsin method.[7, 8] Glycogen could be demonstrated as red-staining material which was dissolved by pretreatment with β amylase at pH 6. Some preparations were treated with water (phosphate buffer pH 7) before denaturation in order to extract the water-soluble mucoprotein. Neighboring sections were also treated with solutions containing trypsin (0.1 mg. per cubic centimeter in phosphate buffer pH 7), pepsin (0.1 mg. per cubic centimeter in 0.01N HCl), collagenase (1 mg. per cubic centimeter in M/15 phosphate buffer pH 7), and testicular hyaluronidase (250 viscosity reducing units per cubic centimeter in M/10 acetate buffer pH 4.5). Appropriate buffer controls were also employed. The technical details of this procedure have been described.[1, 4]

OBSERVATIONS

Histochemical Findings (Figs. 1 and 2).—The connective tissue of normal gingiva is stained pink by the periodic acid-leucofuchsin reagent. The ubiquitous ground substance which appears to surround and coat the collagen may mask the fibers. A general effect of optical homogeneity is thus created in many instances. The basement membrane, which demarcates the epithelial layer from the connective tissue, appears as an undulating, magenta-colored linear structure. The blood vessels, too, stand clear because of the staining of their basement membranes.

After treatment with water (M/15 phosphate buffer pH 7), the pink staining is diminished. Pretreatment with proteolytic enzymes, such as trypsin and pepsin, or with collagenase leads to even greater losses in stainability. These results are largely similar to those obtained by Gersh and Catchpole[4] with skin. It was not possible to determine the effect of hyaluronidase.

When the gingivae are inflamed, the ground substance is more lightly stained. Following extraction with water, the staining is even paler, suggesting the presence of considerably more water-soluble glycoprotein than is apparent in similar sections of normal gingiva.

The basement membrane about blood vessels and that adjacent to the epithelial layer is changed in inflammation. It is either swollen, or more

usually attenuated or thinned. The rather complete dissolution previously described in desquamative gingivitis[1] was only rarely encountered.

Many of the connective tissue cells contain increased amounts of cytoplasmic glycoprotein as well as glycogen (carbohydrate aggregates which could be removed by β amylase).

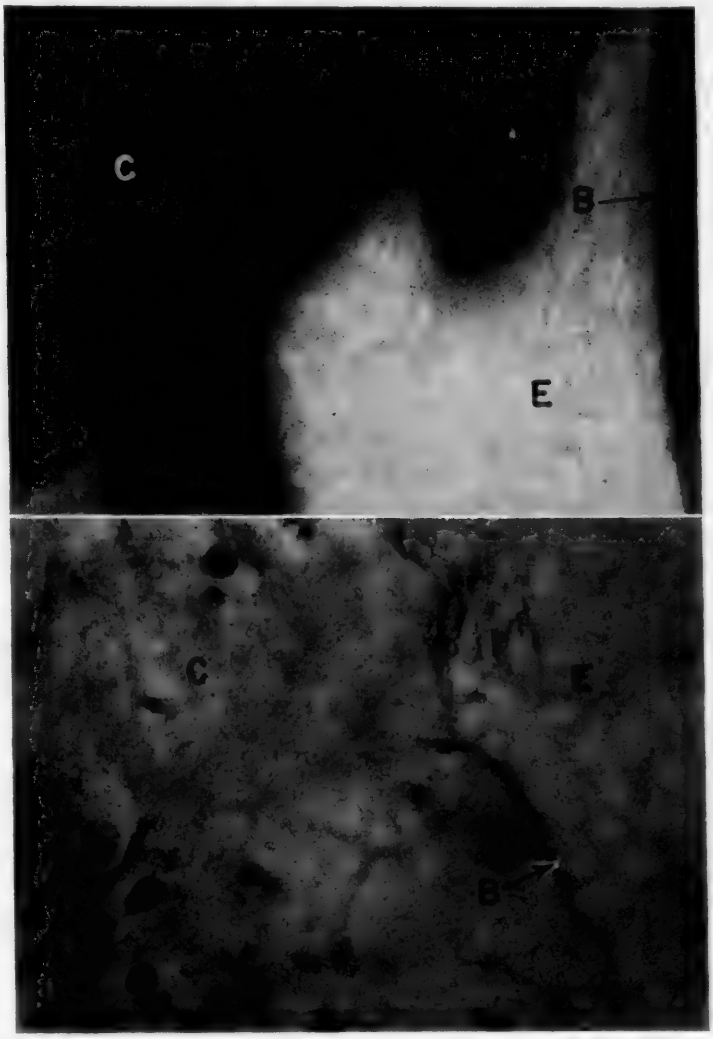

Fig. 1.—Normal gingiva. Connective tissue ground substance is fairly homogeneously stained. Subepithelial basement membrane is well defined.

Fig. 2.—Inflamed gingiva. Ground substance stains less intensely. Basement membrane is considerably thinner than in normal preparations. Sections of gingiva in Figs. 1 and 2 were cut at 6 microns, deparaffinized in xylol, and denatured overnight in 95 per cent ethanol. They were then stained for carbohydrate-containing substances with the periodic acid-leucofuchsin reagents. *B* represents basement membrane, *C* connective tissue, *E* epithelium.

Analytic Results (Table I, Fig. 3).—The water-soluble mucoproteins of the gingiva were determined and expressed in terms of a carbohydrate component (mannose-galactose). For the fifteen normal specimens, the mean value was 254 mg. per 100 Gm. of dry defatted tissue. A mean value of 386 mg. per 100 Gm., a one and one-half fold increase, was obtained from the group of inflamed tissues. The difference between the two groups is a highly significant one, as confirmed by statistical analysis ($P < .01$).

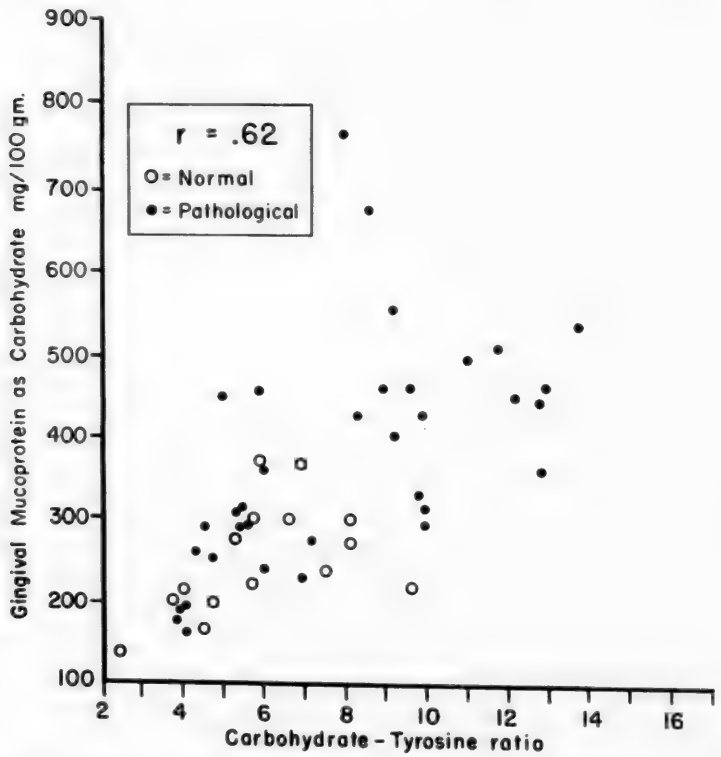

Fig. 3.—Graph showing relationship between carbohydrate concentration of water-soluble mucoprotein of gingiva and carbohydrate-tyrosine ratio.

The tyrosine values for the two groups, 41 mg. per 100 Gm. for the normal and 48 mg. per 100 Gm. for the pathologic tissues, did not differ significantly. The carbohydrate-tyrosine ratio was somewhat greater in the latter case. A graph developed by plotting carbohydrate values against the carbohydrate-tyrosine ratio suggests a positive relation between these two ($r = .62$).

DISCUSSION

The soluble and insoluble portions of the connective tissue ground substance may be thought of as coexisting phases of a colloid.[5, 9] The soluble part would

TABLE I

MEAN VALUES OF WATER-SOLUBLE MUCOPROTEINS OF DRY DEFATTED GINGIVA EXPRESSED AS CARBOHYDRATE AND TYROSINE

	NUMBER OF CASES	CARBOHYDRATE (MG./100 GM.)	P	TYROSINE (MG./100 GM.)	P	CARBOHYDRATE-TYROSINE	P
Normal	15	254 ± 16*	less	41 ± 2.7	greater	6.5 ± 0.46	less
Pathologic	38	386 ± 25*	than 0.01	48 ± 3.7	than 0.7	8.4 ± 0.56	than 0.05

*± indicates standard error of mean.
P values calculated from Fischer's t.

correspond to a water-rich, colloid-poor phase which is in equilibrium with the insoluble colloid-rich, water-poor phase. Under physiologic conditions, the relative quantities of the two phases might fluctuate but the intrinsic composition of each phase is thought to remain constant. Such an equilibrium would permit uptake or release of water and of electrolytes within a variable range without leading to alterations in osmotic pressure of the blood or extracellular ground substance. The stable composition of the colloids is suggested in normal gingival tissue by the limited range of variation in the carbohydrate-tyrosine ratio. Over 86 per cent of the cases ranged from 4 to 8.

In gingival disease where there is swelling and edema, the increased quantity of water-soluble mucoprotein would correspond to an increase in a water-rich, colloid-poor fraction. In such an instance of a pathologic process, a change in the composition of the phases may be signaled by the wide range in the carbohydrate-tyrosine ratio, which varies from 4 to 16 and tends to increase as the water-soluble mucoprotein increases (Fig. 3). Similar increases in water-soluble mucoproteins of the connective tissues have been demonstrated about growing organs and tumors.[4, 10]

The changes in the connective tissue in gingival disease may be caused by bacterial action, they may arise as a part of a generalized response of connective tissues to some constitutional stimulus, or both factors may be involved. Some oral microorganisms elaborate mucolytic and proteolytic enzymes. Under conditions of poor hygiene or of an initially altered tissue state, these bacterial enzymes may act to dissolve the ground substance. On the other hand, in certain states associated with hormonal changes, as during pregnancy or puberty, the tissue responses are related to the internal milieu.[3] Then the increase in the water-soluble mucoprotein might be attributed to the depolymerization of ground substance colloid or to the synthesis of increased amounts of water-soluble mucoprotein, or to both reactions.

SUMMARY

In some pathologic states the connective tissues of the gingiva contain appreciably greater amounts of water-soluble mucoprotein carbohydrate than do normal tissues. This change is attributed to enzymatic depolymerization or to altered synthesis of the connective tissue ground substance.

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A METHOD OF RECORDING MASTICATORY LOADS

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THERE have been many types of apparatus designed for the measurement of biting forces, all depending on the insertion between the teeth of a device which inevitably opens the bite to a variable extent. Such apparatus can only be used statically to determine the range of forces which can be exerted between the jaws, but cannot be used for measuring masticatory loads. One of the most recent of these instruments[1] was small enough to record the maximum load which could be applied between individual teeth in the arch. In this article a method is described by which biting loads can be measured without interfering with the normal occlusion of the teeth. The method is suitable for studying loads achieved during mastication.

METHOD

The method involves the use of the resistance wire strain gauge, which is frequently employed by engineers to record strain in various structures under load. The strain gauge to be described is the standard pattern designed and produced at the National Physical Laboratory, Teddington, and consists of a coil of fine wire wound onto tissue paper impregnated with phenolic resin, the whole being compressed into a flat rectangular sheet. This is stuck firmly to the structure to be tested, and the dimensional changes under load are transmitted to the coil of wire, producing a resistance change. This can be recorded in a Wheatstone bridge circuit, and, over a suitable range of loads, the resistance change is proportional to the dimensional change.

The strain gauge can be used to measure loads if it is fixed to the under-surface of a bar of material supported at both ends and free to bend under a load applied at some point on the unsupported surface. The material of the bar and its thickness must be chosen for the range of loads it is expected to measure, for not only must the dimensional change be kept within certain limits dictated by the extensibility of the gauge wire, but it must be a reversible change. In order to make absolute determinations of the loads applied to such an apparatus, it is necessary to calibrate it with known loads. The out-of-balance deflections on a galvanometer in the bridge circuit provide a means of making static measurements, but by substituting a D.C. amplifier and cathode ray oscilloscope for the galvanometer, the apparatus can be used as a dynamic, as well as a static, recorder.

With the assistance of the Engineering Division of the National Physical Laboratory, which designed and constructed wire strain gauges of dimensions approximately 2×3 mm. and 50 ohms resistance, it was posible to make a load recording unit small enough to be fitted into a cavity in a molar tooth without

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any interference with the normal occlusion. The bar which transmitted the load to the gauge was made of mild steel tempered to straw color, and the final shape of the gauge unit adopted for these experiments is shown in Fig. 1. The unit was placed in a cavity cut in a gold inlay in a lower molar, the position of the cavity being arranged so that a cusp of the opposing tooth occluded normally

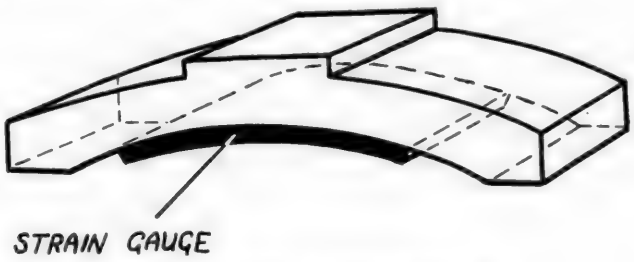

Fig. 1.—Diagram of strain gauge unit.

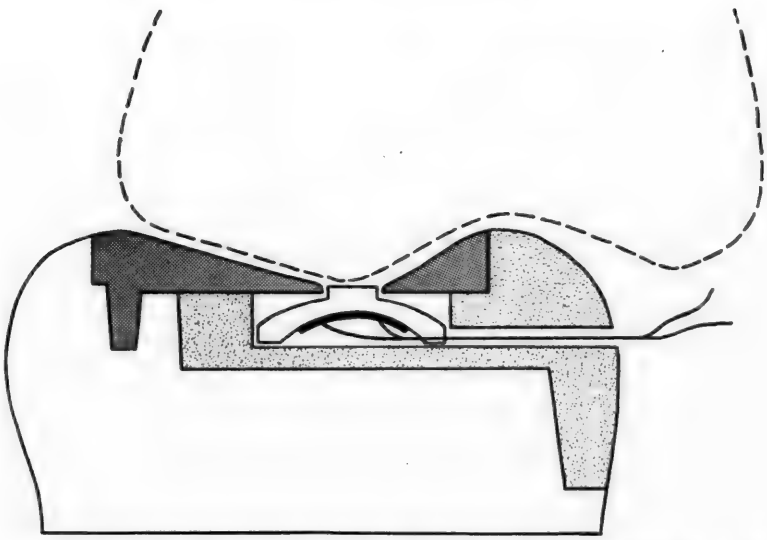

Fig. 2.—Diagram of gauge unit in position. The leads leave through a hole cut in the main inlay (shown lightly shaded). The removable covering inlay is shown darkly shaded.

with the central part of the gauge unit. It was found that the gauge unit functioned satisfactorily only if the load was taken on its central portion while the ends were free. A small covering inlay let into the main gold inlay protected the ends of the unit from pressure by food during chewing while leaving the central elevated portion exposed to take the load. This covering inlay was constructed of hard gold alloy and the clearance between it and the ends of the gauge unit ensured that it transmitted no pressure to the ends of the unit.

The covering inlay was kept in position by virtue of two downward projections into the dentin. The arrangement of the various components is shown in Fig. 2.

Fine wire leads were soldered to the ends of the gauge wire, and both leads and gauge were coated with Bakelite VMCH resin as a protection against moisture. The leads left the tooth through a hole lingually and distally, crossing under the tongue to reach the exterior through the interdental space between incisors or premolars on the other side of the mouth. A small acrylic resin lingual plate behind the lower teeth prevented the lead wires from rising during mastication. The gauge unit was calibrated in an amalgam model of the tooth with known loads. Fig. 3 is the calibration curve for the unit used in the experiments to be reported, the ordinates obtained by direct measurement on the photographic records from the oscilloscope.

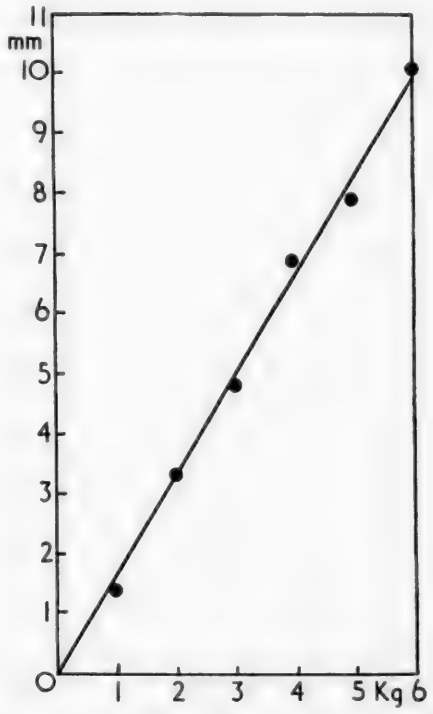

Fig. 3.—Gauge unit calibration curve. The ordinates were obtained by direct measurements on the photographic record from the oscilloscope during the application of known loads to the gauge unit outside the mouth. The abscissas are the loads applied.

RESULTS

Fig. 4 shows a typical series obtained while chewing biscuit. Four pieces of biscuit were chewed and swallowed, each sequence lasting about eleven seconds. It will be seen that the actual recorded load increased toward the end of each sequence. The final peak coincided with closure of the jaws during swallow-

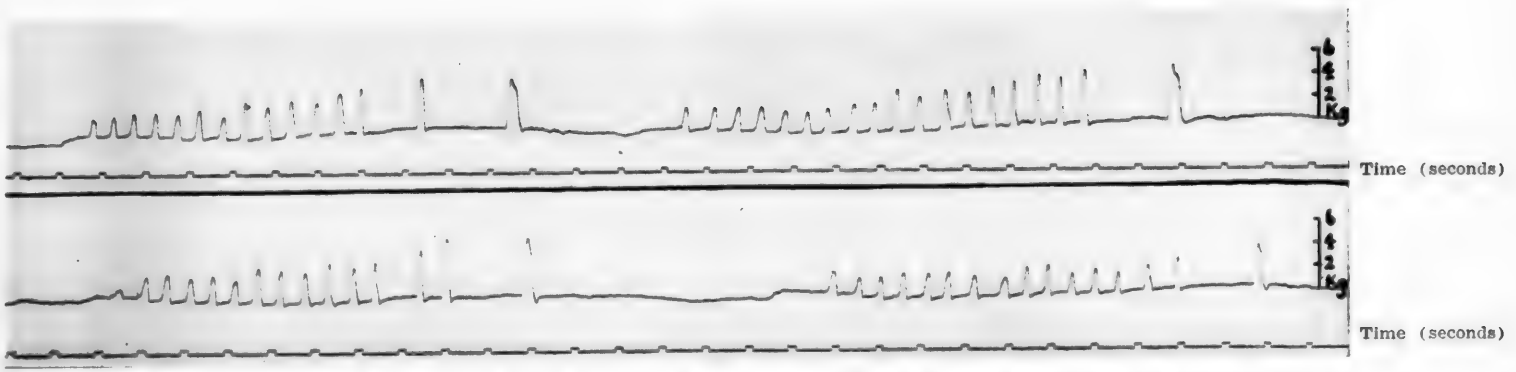

Fig. 4.—Chewing record. Four pieces of biscuit were chewed and swallowed. Time tracing shows one-second intervals.

ing and is a feature of all the records obtained from this subject. The calibration scale is taken from the curve in Fig. 3 and shows the peak loads to be about 4 to 6 kg.

DISCUSSION

The results show that in chewing soft foods such as biscuit, the jaw muscles do not exert the very great forces of which previous static measurements have shown them to be capable. Further work with different food substances is in progress to ascertain physiologic maxima. At present no explanation is offered for the rise in the recorded loads during chewing. Preliminary experiments with another subject suggest that the clenching of the teeth during swallowing is not present in all swallowing patterns, a fact already appreciated by orthodontists.[2]

SUMMARY

1. A method of recording masticatory loads by the use of an intradental resistance wire strain gauge is described.

2. Preliminary results obtained with this method are reported.

The author is indebted to Mr. H. L. Cox and Mr. W. J. Pullen of the Engineering Division of the National Physical Laboratory, Teddington, for the design and construction of the resistance wire strain gauges used in this work. He also is indebted to Dr. L. C. Thomson for the D.C. amplifier and considerable assistance in obtaining results in the early stages. Finally, he is grateful to his colleagues at Guy's Hospital Dental School for the preparative dental work.

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UTILIZATION OF CONSTANT EXPOSURE FACTORS FOR INTRAORAL ROENTGENOGRAPHIC STUDIES

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INTRODUCTION

IN A previous investigation,[1] the radiopacity of oral structures was compared with an ivory step-tablet exposed on intraoral film. It was shown that the density of oral structures varied beyond the limits of 19.5 and 4.5 mm. of ivory, and that these were properly considered the minimal range. It was found that this range could be covered more effectively by utilization of 90 to 95 kilovolt peak (KVP) than with the 42 to 65 KVP customarily employed. At that time, and previously,[2] it was observed that as the kilovoltage was increased with proper decreases in milliampere-seconds (Ma.S.) the full scale of structural densities was seen in spite of considerable variation in the quantity of radiation. This suggested the possibility of constant exposures for all areas of the mouth, and in mouths of patients in different weight and age brackets.

If these observations could be substantiated by a quantitive investigation, it would indicate important practical advantages for the high KVP technic. Possibly all intraoral films could be taken at the same setting on the x-ray machine without regard for differences among patients or positions in the mouth. Observable density differences then could represent tissue density variations unmodified by exposure factors.

EXPERIMENTAL PROCEDURE

As in previous experiments, the x-ray equipment consisted of a shockproof full-wave rectifier having a capacity of 200 Ma. at 90 KVP, or 10 Ma. at 110 KVP. It was equipped with a shockproof transformer, a filament stand-by voltage and amperage stabilizer accurate to plus or minus 5 per cent, a milliampere stabilizer accurate to plus or minus 10 per cent, and a synchronous timer with a range of 1/10 of a second to 14 seconds accurate to twelve plus or minus one impulse per 1/10 second as checked with a spinning top. The x-ray tube was a shockproof, oil immersed, air flow, double focus type with focal spots of 1.5 and 3.8 mm. Only the 1.5 mm. focal spot was used. The output of the machine was 2.82r per second in air at 23 cm. TSD 70 KVP and 10 Ma. with no extrinsic filtration. Under these conditions the half-value layer (HVL) in millimeters of Al was 0.8. The kilovoltage, as recorded on the prereading primary voltmeter calibrated in KVP to include line drop, was checked at the date of installation and following completion of the investigative work. It was found to be both consistent and accurate. In a like manner the line voltage

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was checked. It was 207 volts with no perceptible drop at 10 Ma., a 1.0 volt drop at 50 Ma., and 1.5 volt drop at 100 Ma. These values were obtained at 60 KVP. Two types of cones were used. One was a steel extension cone. This had a minimum length of 10 inches and a maximum length of 17½ inches with 4¼ inch aperture at its extreme end. Although no diaphragm was used with this cone, 0.5 mm. Al filtration was used. The opening of the cone connected to the tube housing was 2¼ inches. The second type of cone used could be adjusted to lengths of 11 inches, 15 inches, and 18 inches, and had an opening of 3 inches at its extreme end. Only the 18-inch length was used with 0.5 mm. Al filtration.

Two types of step-tablets were used. An aluminum step-tablet measuring 9¾ inches in length, composed of nineteen thicknesses of 1 mm. aluminum, was used for extraoral procedures. Each step of the tablet was 1 inch × ⅜ inch in size. A small intraoral ivory step-tablet also was used, which measured 33 mm. in length and 9 mm. in width, with eleven steps measuring 1.5 mm. in thickness, except for the first step which measured 4.5 mm. Ivory was selected because it was reported[3] to have calcium as its principal absorbing element, thus providing a reasonable comparison with dentin and bone and also providing continuity with work previously carried on.[1, 2]

A special densitometer was used. Its light was supplied by five 4 watt "daylight" fluorescent bulbs mounted side by side over a white surface. The bulbs were 5 mm. apart and formed an effective area of 9.5 cm. × 11 cm. The light was diffused by passage through two white translucent Plexiglass plates 3/16 of an inch thick and 1 inch apart. A sheet of translucent paper was placed between the Plexiglass plates with its convexity upward so as to distribute the light equally over the entire upper plate. The degree of convexity needed to give uniform light was determined experimentally. A mask was placed on the surface of the upper plate to cut off all light except that shining from the center of the plate through that portion of each step-tablet to be viewed. A Weston photo cell was attached above this area on a hinged mounting so that the cell closed over the light source in the same position each time. A guide was also made along the edge of the mounting so that all the step-tablet images could be placed in exactly the same position. The photo cell was attached directly to a (0-100) DC micrometer and the readings were taken from the meter. In use, the meter read from 0 to 68 units. This range covered the entire density range from a zero reading for completely black film to a reading of 64 for unexposed film. The maximum reading was 68 when no intermediary substance was placed in the meter.

The film employed was Kodak no-screen film No. 4 and Kodak ultraspeed dental film. Radiatized dental film was not included because of known clinical impracticability at lower kilovoltages. Following the manufacturer's recommendations, the no-screen film was developed 7½ minutes and the ultra-speed film was developed for 5 minutes at 65° F., plus or minus one degree. All films were washed for a minimum of 30 seconds between developer and fixer

in running water and fixed for 15 minutes. Following fixing, the films were washed in running water for 20 minutes. They were processed on hangers, agitated by hand, and dried in air.

RESULTS

The first purpose of this study was to show the effect of Ma.S. and KVP change on the density of a film beneath a step-tablet, to express this effect numerically, and to establish the fact that diagnostic intraoral films can be taken at a single Ma.S. if kilovoltages in the vicinity of 95 KVP were used. Exposures

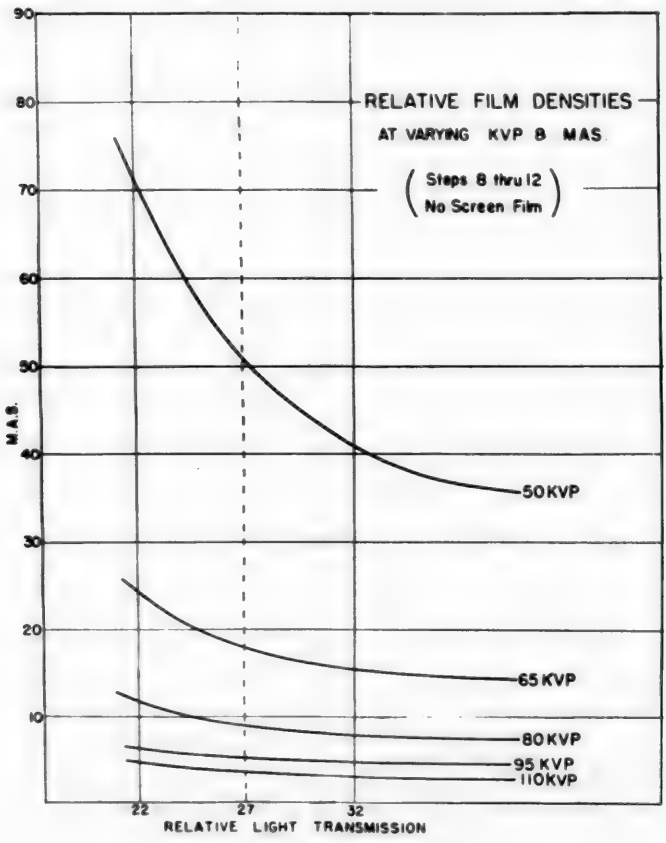

Fig. 1.

of the aluminum step-tablet were made using no-screen film at various KVP-Ma.S. settings. The central beam was directed at right angles to the film and at the center of the 10 mm. step. A two-spot plumb line system with the extension type cone was employed to check the accuracy of the ray direction. The target-to-film distance was 36 inches. The resultant films were measured for total light transmitted through steps 8, 9, 10, 11, and 12. Five steps were used instead of the full tablet in order to minimize error introduced by the

anode-heel effect. The meter readings (light transmission) are plotted against the Ma.S. for several KVP levels in Fig. 1. It was found that the light transmission of films taken at these factors is reproducible within 3 per cent over the range of exposures studied.

It seemed important to know those factors which at different KVP levels would produce a "common" light transmission. Previously,[2] using a 36-inch TFD and no-screen films, 95 KVP and 8 to 9 Ma.S. had been shown to be somewhat excessive for demonstrating soft tissue. Six Ma.S. was felt to be optimum for this purpose. The light transmission of steps 8 through 12 of the step-tablet, taken at 95 KVP and 6 Ma.S., was accepted as the "common" transmission (meter reading of 27). The dotted line in Fig. 1 demonstrates the common transmission readings and the Ma.S. values at various KVP's which produce the same amount of light transmission.

It was observed that the per cent of Ma.S. change necessary to produce a given density other than the common density is lower in the high KVP range than in the low KVP range. Table I shows the per cent of Ma.S. change necessary to maintain constant film densities through steps 8 to 12 for points on either side of the common transmission line. A meter reading of 5 units on either side of this line was arbitrarily selected to demonstrate the preceding. The percentage of Ma.S. change for ten units of light transmission in Table I (A-C), is obtained by adding the per cent of Ma.S. change necessary to produce 5 units of light change on either side of the common transmission.

TABLE I

	METER READING								
	22 Ma.S.*	27 Ma.S.*	32 Ma.S.*						
KVP	A	B	C	A-B	%	B-C	%	A-C	%
110	5	4	3½	1	25	½	12.5	1½	37.5
95	7½	6	5½	1½	25	½	8.3	2	33.3
80	12	9	8	3	33.3	1	11.1	4	44.4
65	25	18	16	7	38.9	2	11.1	9	50.0
50	71	51	41	20	39.2	10	19.6	30	58.8

*The Ma.S. required to produce the above meter reading at appropriate KVP.

Ma.S. is ordinarily varied to compensate for changes in tissue density on either side of an average. Changes in tissue density, in order to be satisfactorily represented, require greater percentage change of Ma.S. at low kilovoltages than at high kilovoltages. Stated in another manner, a greater span of tissue densities can be observed at high kilovoltages than at low kilovoltages as a result of an equal percentage change in Ma.S. It is known that if a constant Ma.S. factor is used for each KVP level, a broader band of tissue densities can be observed at high kilovoltages than at low kilovoltages. It will be noted from Table I that the percentage of Ma.S. change for ten units of light transmission at 95 KVP is approximately one-half the per cent of Ma.S. change for ten units of light transmission at 50 KVP and approximately 40 per cent less than the per cent of Ma.S. change for an equal number of units at the commonly used 65 KVP. These figures suggest that at a constant Ma.S. it is possible to

demonstrate a 40 to 50 per cent greater range of tissue density at 95 KVP than at the lower potentials. The possibility of obtaining a complete oral survey at a fixed Ma.S. and a low KVP has been demonstrated. The films are reasonably diagnostic, but range from heavy to light densities which are often beyond pleasing limits. It has also been established clinically and in a previous investigation,[1] that the Ma.S. used is critical. Since a 40 to 50 per cent greater range of tissue density is possibly at 95 KVP, it becomes apparent that the tendency for extremes in film densities and the necessity for as critical an Ma.S. would be diminished. Since the slope of the curves of the higher kilovoltages in Fig. 1 tends to reach a minimum, it seems reasonable to suppose that no further advantage could be gained by kilovoltages in excess of 95 KVP.

TABLE II

EXPOSURE RANGES KNOWN TO GIVE EXCESSIVELY LIGHT AND EXCESSIVELY DARK FILM AT THE EXTREME

NO-SCREEN FILM (EXTRAORAL)					
110 KVP Ma.S.	95 KVP Ma.S.	80 KVP Ma.S.	65 KVP Ma.S.	50 KVP Ma.S.	35 KVP Ma.S.
1	1	1	2	3	8
2	*2*	*3*	*5*	7	16
3	4	5	8	*11*	24
4	6	7	11	*15*	*32*
5	8	9	14	19	*40*

TABLE III

EXPOSURE RANGE KNOWN TO GIVE EXCESSIVELY LIGHT AND EXCESSIVELY DARK FILM AT THE EXTREMES

ULTRA-SPEED FILM (INTRAORAL)					
110 KVP Ma.S.	95 KVP Ma.S.	80 KVP Ma.S.	65 KVP Ma.S.	50 KVP Ma.S.	35 KVP Ma.S.
2	2	5	10	20	50
4	5	10	20	40	100
6	*10*	*20*	*30*	60	150
8	*15*	*30*	*40*	*80*	*200*
10	20	40	50	100	250

The second purpose of this study was to establish the optimum Ma.S. for intraoral ultra-speed films taken at 95 KVP. Exposures were made of the ivory step-tablet at a 23-inch target-to-film distance using no-screen film cut and packaged to the size of dental film. The 18-inch cone was used. This distance was selected since it was the approximated TFD planned for the intraoral experiment and future clinical work. Exposures were made at 95 KVP through an Ma.S. range which would give both excessively light and dark film. Five observers familiar with reading radiographs each chose what he considered to be the most satisfactory film. From the relative sensitivity[4] of no-screen film and ultra-speed film, it was possible to transpose the factors for experimental intraoral use of ultra-speed film. Adjustments in exposure were made for the density of the cheek tissue. Several intraoral exposures were made, using ultra-speed film with the ivory step-tablet placed sidewise on the occlusal surface of the teeth. The resultant films were compared, by means of a Weston Model 877

light meter, with the most satisfactory step-tablet taken on no-screen film. The optimum Ma.S. value derived in this manner was between 10 and 15 Ma.S. Table II shows the Ma.S. values used in this determination and, for reference purposes only, also shows the Ma.S. values necessary to produce excessively light and dark films at additional KVP levels. The italicized values at each KVP of Table II were chosen by five observers to be optimum for that KVP. The italicized values in Table III are those selected for intraoral use with ultra-speed film as being comparable to the values in Table II. Where two values are italicized the preferred value lies intermediate.

In practice it has been possible to produce diagnostic intraoral films in adults and children, using a constant 13.75 Ma.S. at 95 KVP, 23 TFD, and ultra-speed film with 0.5 mm. of Al filtration. Standardized developing procedures, as outlined previously, were employed.

SUMMARY

The purpose of this study was to evaluate the possibility of using a constant Ma.S. value at kilovoltages in the vicinity of 95 KVP to produce diagnostic intraoral x-ray films, and to suggest a suitable exposure value for this procedure. It was found that at 95 KVP the per cent of change in Ma.S. required to produce equal film densities on either side of a common density was less than at any other KVP, from 110 to 50. This fact establishes the original premise that at a constant Ma.S. a greater range of intraoral tissue densities may be seen at 95 KVP than at any other KVP within the range studied. This range of tissue density is sufficient to allow for the production of diagnostic radiographs in patients having considerable variation in bodily structure. An exposure value between 10 and 15 Ma.S. was shown to be optimum for intraoral films using a 23-inch target-to-film distance, 0.5 mm. Al filtration, ultra-speed film, and a peak kilovoltage of 95 under standard developing procedures.

The theoretical considerations suggested have been substantiated on a clinical basis.

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THE EFFECT OF SINGLE AND FRACTIONATED DOSES OF SELECTIVELY APPLIED X-RAY IRRADIATION ON THE HISTOLOGIC STRUCTURE OF THE MAJOR SALIVARY GLANDS OF THE RAT*

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THE clinical effects of roentgen-ray irradiation on oral and para-oral structures have been recognized for many years. Two particular phenomena of extreme interest in patients whose salivary glands have been accidentally or purposely irradiated are the xerostomia and the peculiar type of tooth destruction which originates usually in the cervical areas of the teeth and spreads rapidly to include the entire crown. That these two characteristics probably have more than a casual relationship is indicated by a similar phenomenon occurring in children with congenitally absent salivary glands, namely, a very rapid carious destruction of the teeth.

The particular problem of irradiation xerostomia is often more than a mere annoyance to the patient. The loss of salivary flow results not only in the loss of such physical properties as the demulcent effect, the "flushing" action, and the "lubricating" action on foods, but also in the probable loss of salivary enzymes and antibacterial substances.

The purpose of the present study has been to attempt to determine the effect of x-ray irradiation on the structure of normal rat salivary glands with the view of correlating this with the known physiologic dysfunction. In order to eliminate as much as possible the systemic effect of irradiation on the salivary glands, these structures were *selectively* irradiated, in contrast to most other studies where total body irradiation was employed and where the salivary glands were studied only incidentally.

Although there have been numerous studies dealing with the effects of irradiation on the teeth and jaws, most of these are superfluous to the present study and no attempt will be made to review these reports. Only those studies relating to changes in the salivary glands following irradiation will be considered here.

Early in the history of radiotherapy, abundant clinical evidence of dysfunction of the salivary glands following irradiation was accumulated. Little controlled scientific evidence was collected, however, until the studies of Ivy, Ordorff, Jacoby, and Whitlow in 1923.[1] Utilizing dogs, these authors attempted to correlate the physiologic activity of the irradiated submaxillary gland with

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histologic changes. By salivary fistulas a reduction in salivary flow was noted, although with moderate doses of irradiation they found no reduction in the volume of blood flowing through this organ. A reduction in total solids and organic solids of the saliva was found. Histologically, no immediate changes in structure were seen, even after large doses of irradiation. Delayed effects did occur, however. After fifteen days there was an infiltration of round cells around the secretory ducts and blood vessels and into the interstitial tissue with some fibroblastic proliferation. The acini appeared to show a reduction in the amount of stored mucus. After a two-month period, the fibrous connective tissue stroma was increased and the acinar cells appeared loaded with stored mucus.

The histologic changes noted by Ivy, Ordorff, Jacoby, and Whitlow appear minimal, considering the moderately sharp reduction in activity and the dosage of irradiation administered. However, as Loeb early pointed out, the sensitivity of cells is apparently related to their proliferative activity and, whereas thyroid and liver are moderately sensitive, the kidney, pancreas, and salivary glands are relatively resistant.[2]

A study by von Salis has pointed out in more detail the histologic alterations occurring in irradiated salivary glands of the dog and in addition has described the changes seen in a limited number of clinical patients.[3] In these human beings, von Salis described granular changes in the submaxillary acini in addition to vacuole formation, nuclear clumping, and some inflammatory edema. In time these irradiated glands apparently returned to their normal structure. Experimentally irradiated dogs showed only slight changes in spite of very intensive treatment (8 to 12 times the usual human dose). In the submaxillary gland spotty degeneration corresponding to cloudy swelling was noted. The secretory ducts were unaffected. Congestion of the capillaries was seen, as well as a nonspecific inflammatory exudate. Similar minimal changes were described in the parotid gland. Two significant conclusions were drawn by von Salis from this study. He reasoned, first, that the chief changes were of vascular origin and, second, that whereas there is degeneration of specific connective tissue elements, they are not replaced by scar tissue. Therefore, he felt that the changes observed are not of a permanent nature.

Case and Boldyreff have also shown that in dogs with experimentally irradiated salivary glands and permanent salivary duct fistulas, there is a marked decrease in the total volume of both stimulated and unstimulated saliva secreted.[4] They found that this diminution of function reached its maximum about three to four weeks after treatment and then slowly returned to complete normalcy after approximately five months. A diminished sensibility of the oral mucosa was also noted.

Tsuzuki, studying the effects of irradiation in rabbits, has reported that the salivary gland acinar cells showed some atrophy after treatment corresponding to a 42 per cent erythema dose.[5] Changes were also described in the epithelial cells of the smallest excretory ducts following a 50 per cent erythema dose, namely, fatty degeneration. In contrast, Jungling has described complete disappearance of gland acini in nine weeks following the application of a 100 to

120 per cent erythema dose, although he noted no changes in the excretory ducts.[6]

Lazarus-Barlow, after exposing rats to radium bromide, has reported the effect of gamma rays on the salivary glands.[7] Six and nine days after irradiation, the most marked changes were seen in the mucous glands, there being little alteration in the structure of the serous glands. These mucous gland acini were swollen and the cytoplasm exhibited a hyaline and granular or mucoid appearance. The nuclei of these cells were irregular and swollen. In a similar study on rabbits the only alterations noted in the salivary glands were early mucoid changes, the occlusion of some ducts by mucous plugs, and some swelling of the columnar epithelium of the secretory ducts.

The reviews by Desjardins[8] and Friedman[9] of the limited number of pertinent studies have served only to emphasize our relative lack of knowledge of alterations occurring in irradiated salivary glands.

English and Tullis,[10] utilizing swine exposed to 450 to 500 r. total body irradiation, demonstrated changes in the salivary glands of the animals which were found dead. However, it was not certain which of these were due to irradiation and which to autolysis. In sacrificed animals there appeared to be some focal destruction of acini and, in the mucous cells, a reduction of the basophilic granules. In those animals which survived for over thirty days and were sacrificed there appeared to be little change except for some increase in cells, suggestive of mucous cells between the acini and occasional areas of fibrosis. In general, any previous damage appeared to have been repaired.

Urban[11] and Schlack and Ellinger[12] have reported the effects of irradiation on experimental dental caries in the rat. The study by Urban has compared the effects of salivary gland irradiation and extirpation. The results were similar, namely, an increased amount of caries as compared to controls, although both the xerostomia and the caries were more severe in extirpated animals than in irradiated animals. An interesting observation was the finding of considerable caries in the gingival areas. As has been pointed out previously, this is a characteristic finding in the human being whose salivary glands have been purposely or inadvertently radiated. It is, however, an unusual area for the development of experimental caries in rats. Schlack and Ellinger, employing only a single 200 r. total body dose of irradiation, have also shown a similar statistically significant increase in experimental dental caries incidence. This study is of considerable interest, since it indicates that something more than salivary gland dysfunction may be involved here in altering the caries incidence.

EXPERIMENTAL METHODS

The albino rats used in this study were all of the Carl Wilson strain* originally obtained from the Wistar Institute. Only male animals were used and, except for those rats in the preliminary studies, all were young animals weighing 100 to 140 grams. The experimental animals were kept in both wire-bottom cages and solid cages with shavings for bedding. The diet consisted of Purina rabbit pellets and water ad libitum.

*Carl Wilson, Beech Grove, Ind.

The radiation factors were kept constant throughout the entire study. A standard Westinghouse superficial x-ray therapy machine with a peak kilovoltage of 150 kv. was utilized. At 140 kv. and 8 Ma., using a 1 mm. aluminum filter and a target distance of 30 cm., 109 roentgen units per minute were delivered. This 109 r. per minute represents the dose in air, the calculated skin dosage being approximately 110 per cent of this amount.

The irradiation was accomplished with the animals under Nembutal anesthesia administered intraperitoneally. With an animal lying on its back, the 2.5 cm. cone of the machine was brought down vertically until it just touched the skin of the neck overlying the salivary glands.

In the first portion of this study the animals were treated with a single dose of irradiation; that is, the total dose was administered in one application. In the second portion of the study, the irradiation was administered over a protracted period of time. By this fractionated treatment, it was found possible to attain a much larger total dosage without experiencing the high mortality incident to single-dose irradiation.

All animals were sacrificed twenty-one days after the day of the last treatment. At sacrifice, the major salivary glands were dissected out in toto and fixed in Helly's solution for twenty-four hours. After paraffin embedding, sections were cut at 7 microns and stained with: (1) hematoxylin and eosin; (2) modified Mallory's connective tissue stain; (3) phosphotungstic acid-hematoxylin; and (4) basic fuchsin-hematoxylin-light green.

RESULTS

A. *The Effect of Single-Dose Irradiation.*—

1. Radiation: 480 r., 500 r., 960 r., 1,000 r., 1,800 r., and 2,000 r. Forty-three experimental animals; twenty-eight control animals.)

All animals survived the twenty-one-day postirradiation period. At the time of sacrifice, all of the experimental animals in the 1,800 r. and 2,000 r. groups exhibited epilation, ranging from mild to severe, over the area of the neck covered by the 2.5 cm. cone. An occasional animal also showed superficial ulceration in this area.

Histologic examination revealed no alterations in the structure of submaxillary, sublingual, or parotid glands of any of the animals in these groups.

2. Radiation: 2,200 r. and 2,400 r. (Six experimental animals; four control animals.)

All animals but one in this group survived the twenty-one-day postirradiation period. At the time of sacrifice, the epilation just described was seen in all experimental animals.

Histologically, there were no significant alterations in the submaxillary glands. However, in one animal there were changes in the sublingual and parotid glands. The sublingual gland showed a marked diminution in size of the mucous alveoli and their mucus content stained very faintly with basic fuchsin in the contrast to the control animals. In addition, the small serous cells at the base of the alveoli exhibited marked proliferation taking the pattern of demilunes, a characteristic finding in the human being, but not in the rat.

These serous cells had an extremely granular cytoplasm and in many acini had caused displacement of the individual mucous cells. No changes in the intralobular ducts were noted.

The serous parotid gland exhibited mild inflammatory and degenerative changes. The small capillaries accompanying many of the intralobular ducts were engorged, and edema fluid separated many of the individual acini. Occasional inflammatory cells were noted, but they were not present in any significant numbers. In addition, most of the acinar cells appeared somewhat enlarged, their cytoplasm was extremely granular, and in some instances only "ghost" nuclei remained. These changes were suggestive of albuminous degeneration. The epithelium of the ducts also showed the latter changes in many cases.

3. Radiation: 3,000 r. (Fourteen experimental animals; thirteen control animals.)

In this group, only ten of the fourteen animals survived the twenty-one days of postirradiation period. All animals exhibited epilation.

A marked variation in response among the irradiated animals of this group was found. Whereas some animals exhibited a purely inflammatory reaction in the salivary glands, other animals showed degenerative changes with few inflammatory changes.

In the mucous sublingual gland, alterations in structure produced by irradiation were minimal. Occasionally there appeared to be a very mild proliferation of the serous cells at the base of the mucous acini, but no changes were noted in the size or shape of the mucous cells nor in the intensity with which these cells were stained by basic fuchsin. There was no evidence of edema, inflammatory cell exudate, or fibrosis in the sublingual gland.

The submaxillary glands of some animals exhibited no alteration in structure as compared to control animals. In some instances, however, there was congestion of the small intralobular capillaries and, in one animal, almost complete obliteration of many acini by an inflammatory cell exudate. In general, there were no marked, consistent changes which occurred in this gland. The intralobular ducts appeared unaffected.

The parotid gland showed the most marked effects of irradiation. In some animals, the acini were widely separated by an inflammatory exudate of cells and fluid, as well as young collagenous fibrils. The cytoplasm of the acinar cells appeared granular and the individual cell membranes were indistinguishable. The nuclei, however, showed no lytic or pyknotic changes in most instances. In other animals, there was no inflammatory exudate present, but instead there was marked swelling of the acinar cells with the cytoplasm assuming an extremely granular appearance and staining more hematoxyphilic. The nuclei of these cells were compressed against the base and appeared quite pyknotic (Figs. 1 and 2). Occasional animals showed changes similar to those just described but of a milder degree, and in these cases numerous mitotic figures were noted in the acini. In these animals the reparative stage appeared to predominate.

4. Radiation: 3,500 r. (Six experimental animals; two control animals.)

In this group, only two of the six experimental animals survived the twenty-one-day postirradiation period, death occurring at varying intervals following treatments.

The histologic changes were similar to those previously described in animals receiving 3,000 r. In the two surviving animals, inflammatory cell infiltration was noted in both the sublingual and parotid glands. as well as mild proliferation of the serous cells in the sublingual gland. The submaxillary glands appeared relatively unaffected.

B. *The Effect of Fractionated Dose Irradiation.*—In the second portion of this study, the animals received their total irradiation in fractionated doses instead of a single dose, radiation factors remaining identical.

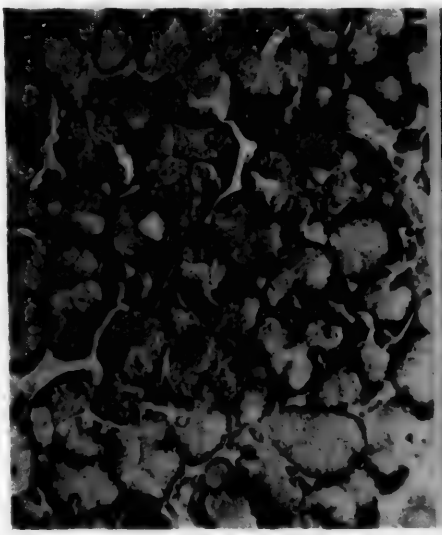

Fig. 1.

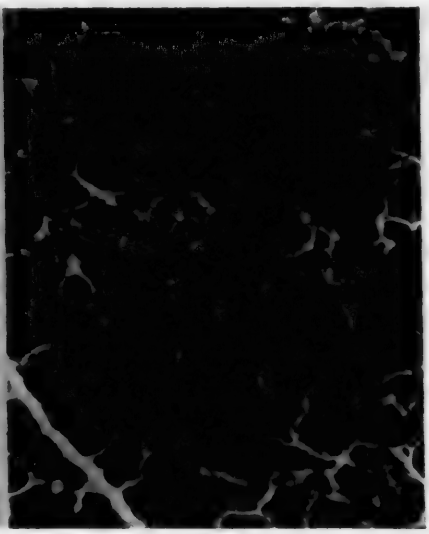

Fig. 2.

Fig. 1.—Parotid gland of an animal receiving 3,000 r. in a single dose. The acini are enlarged, the cytoplasm shows marked granularity, and the nuclei are compressed against the base of the cells.

Fig. 2.—Parotid gland of a control animal.

1. Radiation: 4,500 r. (Six experimental animals; six control animals.)

The animals in this group were irradiated at three-day intervals for one week and then at weekly intervals for an additional three weeks with 750 r. at each treatment. The irradiation period extended over an interval of twenty-nine days and the animals were sacrificed twenty-one days following the last dose of irradiation. Five of the six treated animals survived the experimental period of fifty days.

At sacrifice, all animals exhibited moderate to severe epilation of the irradiated areas. In addition, the salivary glands appeared smaller than those of the control animals.

Histologic examination revealed a generalized progression of the alterations noted in animals receiving a single high dose of irradiation. The submaxillary gland was relatively unaffected. The acinar cells, as well as the structure of the acini themselves, exhibited little, if any, deviation from the normal. Similarly, there were no changes noted in the secretory ducts.

The sublingual gland exhibited a variety of moderately severe histologic alterations. In some instances, the acini were markedly reduced in size and, while the individual acinar cells were considerably smaller than normal, they exhibited no variation in staining intensity with basic fuchsin as compared to the controls. A mild proliferation of the basal serous cells was also a common feature in this group. All animals showed a mild interstitial infiltration of chronic inflammatory cells in this gland, and in some animals there was an appreciable amount of new fibrous connective tissue found between the acini. The secretory ducts were essentially normal.

The parotid gland also appeared to show structural changes. The acinar cells exhibited the granular-appearing cytoplasm which was noted previously in animals subjected to single-dose irradiation, and the nuclei were often compressed against the periphery of the cell. It was common for a mild infiltration of chronic inflammatory cells to be found between the acini, as well as considerable fibrous connective tissue. The secretory ducts appeared unaffected.

2. Radiation: 5,000 r. (Five experimental animals; three control animals.)

The animals in this group were irradiated twice a week for five weeks with a dose of 500 r. at each treatment. The irradiation period extended over an interval of thirty days and all animals were sacrificed twenty-one days following the last day of irradiation. Four of the five treated animals survived the experimental period of fifty-one days.

Histologically, the changes noted were similar to those just described under animals exposed to 4,500 r. in fractionated doses, although none of these alterations were of any increased severity. The inflammatory reaction was minimal and little fibrosis was noted in the sublingual gland in contrast to that seen in the 4,500 r. fractionated dose group.

3. Radiation: 8,000 r. (Six experimental animals; three control animals.)

The animals in this group were irradiated twice a week for five weeks, then once a week for the following six weeks with a dose of 500 r. at each treatment. The irradiation period extended over an interval of sixty-four days, and all animals were sacrificed twenty-one days following the last day of irradiation. Four of the six experimental animals survived the experimental period of eighty-five days.

Histologic examination revealed more pronounced alterations in structure than was seen in any of the irradiated groups previously described.

The submaxillary gland, despite the severe changes manifested in the sublingual and parotid glands, remained relatively unaffected. Any deviation from the normal structure was so slight that it could not be detected by means at our disposal.

The sublingual gland exhibited a reduction in size of the mucous acini. There was no decrease in the intensity of the tinctorial reaction with basic

fuchsin as compared to control animals, however. The peripheral serous acini appeared prominent in most animals, but did not seem markedly increased in number. The outstanding characteristic appeared to be the fibrosis which was evident between the individual acini. Numerous young collagenous fibers and fibroblasts were prominent here, as well as a considerable number of chronic inflammatory cells. This fibrosis had caused spreading of the acini and apparently some compression (Figs. 3 and 4). There did not appear to be any significant increase in the number of capillaries in these interstitial areas. The epithelium of the intralobular ducts exhibited no changes, nor did the blood vessels show any deviations from their usual structure, although occasional mucous plugs in the ducts were seen.

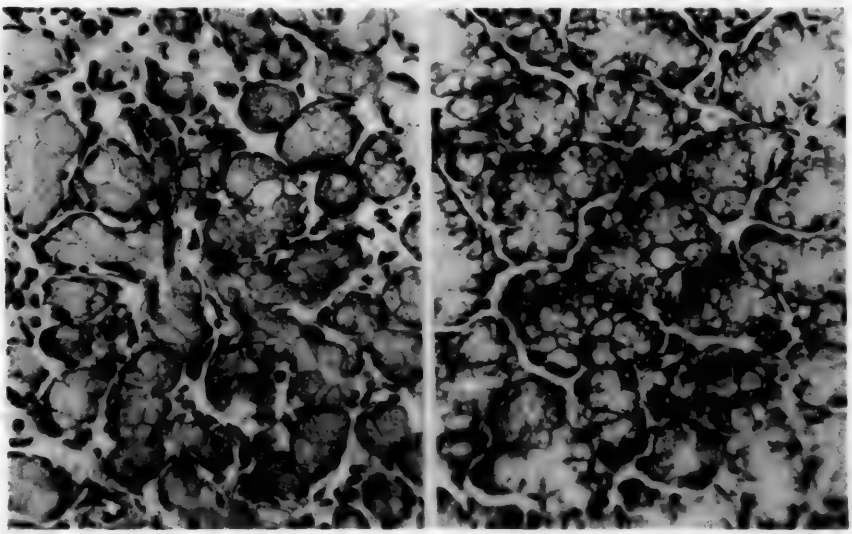

Fig. 3. Fig. 4.

Fig. 3.—Sublingual gland of an animal receiving 8,000 r. in a fractionated dose. There is a decrease in size of the mucous acini and the interstitial areas show infiltration of inflammatory cells and early fibrosis.

Fig. 4.—Sublingual gland of a control animal.

The parotid gland changes were manifested by a moderate decrease in the size of the individual acini, a granular appearance of the cytoplasm in the acinar cells, and obliteration of the cell membranes of many of these acinar cells. Nuclear changes were not prominent. In addition, there was marked interstitial fibrosis, the young collagen fibers being distributed between the individual acini and intermingled with large numbers of chronic inflammatory cells (Figs. 5 and 6). There appeared to be no significant changes in the secretory ducts, and the blood vessels present were materially unaffectd.

DISCUSSION

The present study was designed to attempt to correlate the known physiologic dysfunction of the salivary glands following irradiation with the histologic

alteration which, it was felt, must occur. Two methods of treatment were utilized: single-dose irradiation and fractionated dose irradiation. In single doses, various quantities of irradiation ranging from 480 r. to 3,500 r. were administered and all animals were sacrificed twenty-one days following treatment. The animals in these groups showed increasingly severe alterations in the structure of the salivary glands characterized by inflammation (inflammatory cell infiltration, congestion, and edema) and degeneration of the parenchymal cells suggestive of albuminous degeneration. It may be of significance to note that, while the sublingual and parotid glands appeared equally affected, the submaxillary gland was extremely resistant to irradiation as measured by actual changes in the histologic structure of the gland under the conditions of this experiment.

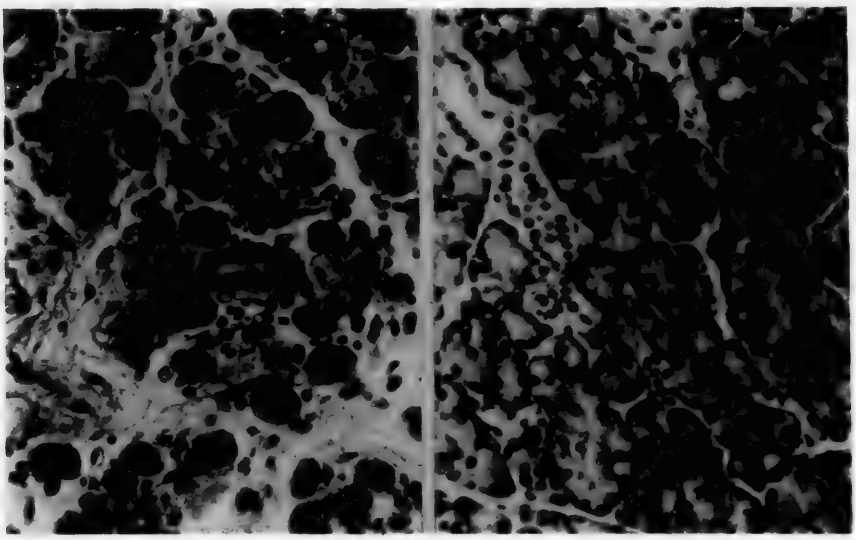

Fig. 5. Fig. 6.

Fig. 5.—Parotid gland of an animal receiving 8,000 r. in a fractionated dose. Although there is some decrease in size of the acini, the chief feature is the edema and inflammatory cell infiltration.

Fig. 6.—Parotid gland of a control animal.

Since it was found impossible to irradiate these animals with over 3,500 r. and still have an appreciable number of survivors, a second method of treatment, fractionated dosage, was employed. It will be realized that this method of irradiation more closely approximates the type of treatment administered to human patients suffering from malignant disease. Any changes noted in the animals under this type of irradiation might be more similar to those which probably occur in the human being following irradiation of the salivary glands. In those animals so treated, the changes seen were merely progressive forms of those alterations following single-dose irradiation. Just as in this latter type of treatment, the submaxillary gland remained relatively refractory to even large

doses of irradiation. The significant changes in the sublingual and parotid glands were of an inflammatory nature, although degeneration with minimal inflammatory changes was also seen. Fibrosis was the most severe condition noted.

It should be pointed out that in some instances it was impossible to determine whether the extremely granular appearance of the cytoplasm of many of the acinar cells indicated a degenerative condition of the cell or an increased storage of secretory granules which might signify a state of functional rest of these cells. The histologic methods utilized in this study were not of such acuity that these two conditions could always be separated.

In general, it may be stated that under the conditions of the study, regardless of the type or quantity of irradiation administered, no changes could be demonstrated which were permanent and irreversible. It is felt that these changes are not compatible with the degree of dysfunction which occurs following irradiation, although it is regrettable that function could not be measured in the animals of this study. It is not unusual for disparity to exist between the alterations of structure and function of an organ or tissue, a fact of which histologists and physiologists are now aware. However, with newer methods and technics in histologic procedures, it is entirely probable that these divergencies may be reconciled. It is suggested, therefore, that although structural alterations observed in this study were of a minor character, histochemical studies using other methods might reveal marked cellular alterations which cannot be detected by the methods used here.

CONCLUSIONS AND SUMMARY

1. The effects of single and fractionated doses of locally applied x-ray irradiation on the histologic structure of the major salivary glands of the rat have been studied.

2. With single doses of irradiation varying from 480 r. to 3,500 r., alterations characterized by increasingly marked degrees of inflammation and degeneration were noted.

3. With fractionated doses of irradiation varying from 4,500 r. to 8,000 r., the alterations noted were merely more severe forms of the inflammatory and degenerative conditions seen following single-dose irradiation.

4. No histologic changes could be demonstrated which were not reversible in type.

5. It is not felt that the minor structural alterations are compatible with the known physiologic alterations, and it is suggested that studies utilizing refined histochemical technics may help clarify this disparity.

The author wishes to thank Dr. John A. Campbell, Associate Professor and Director of the Department of Radiology, Indiana University School of Medicine, and the members of his staff for their help and consultation in many technical, clinical, and theoretical aspects of this problem.

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THE INCIDENCE OF DENTAL CARIES IN HAMSTERS RECEIVING TWO DIFFERENT WATER-SOLUBLE FLUORIDES AT LOW CONCENTRATIONS

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PREVIOUS work has demonstrated the superiority of stannous fluoride over sodium fluoride in reducing the incidence of dental caries in the rat when both materials are administered at similar fluorine levels in the food[1] or drinking water.[2] In one experiment[2] two different strains of rats were used with similar results in each strain. In all such experiments using the rat wherein a comparison between fluoride compounds was made, a coarse corn diet was used as the cariogenic agent. Since some criticism has been suggested against comparing caries in the human being with caries in the rat under these conditions, many workers have used the hamster as the investigative animal because this rodent will produce caries on a soft, fracture-free diet. Further use of this animal in dental caries studies is rationalized on the basis that the hamster's fully covered enamel cusps make the dentition more similar to the dentition of man. For these reasons, this study was designed to investigate the caries-reducing effect in the hamster of two soluble fluoride compounds administered at low fluoride concentrations in the drinking water. The amount of fluorine stored in the skeletal system was also measured to determine whether or not any difference exists in the fluorine deposited from each compound.

EXPERIMENTAL

A total of twenty-one female hamsters were bred and the litter mates of each sex were equally distributed into three groups. One group received stannous fluoride and another received sodium fluoride in the drinking water, each at a fluoride concentration of 10 micrograms per milliliter, while the third was a control group receiving distilled water. All three groups received a cariogenic diet ad libitum consisting of the following: ground whole wheat flour, 30 per cent; cornstarch, 20 per cent; confectioners' sugar, 15 per cent; whole powdered milk, 30 per cent; alfalfa meal, 4 per cent; sodium chloride, 1 per cent. The animals were housed in an air-conditioned room in raised metal cages with fluorine-free filter paper for bedding. The supplements and the cariogenic diet were started when the hamsters were 30 days of age and the experimental period lasted ninety days.

At the end of this period the heads were removed for dental caries studies and placed in 10 per cent formalin until ready for examinations, at which time

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they were stored in 50 per cent ethyl alcohol. The femurs were removed, dissected of soft tissue, and analyzed for fluorine, calcium, and phosphorus.

CARIES SCORING METHOD

The method used for indicating the severity of caries attack is referred to as S.I. (severity index). It is obtained by making a one-step severity judgment of each tooth and totaling the scores in each animal. This method is less tedious and avoids some of the discrepancies which arise in grading on a cavity basis. At the same time S.I. gives more information about the dental condition than a simple count of the number of cavities or the number of carious teeth. This consideration is important if one desires to record the dental seriousness of the condition resulting from caries attack. The S.I. method is an attempt to satisfy requirements for simplicity and dental validity.

The procedure for determining S.I. involves the use of a wide-field binocular microscope at a magnification of 15 diameters. Each molar is viewed at several angles to arrive at an estimate of the proportion of the crown which is decayed. S.I. scores from 0 to 3 are assigned to each molar on the following bases: no decay, 0; less than one-fourth of the crown decayed, 1; one-fourth to one-half of the crown decayed, 2; more than one-half of the crown decayed, 3. The observer is required to exercise some discretion in scoring, but he has the advantage of being able to sum up the dental seriousness of all the decay in each molar while viewing the molar directly. Making such judgments from sketches or charts is an indirect approach which may involve additional error. With very little practice an observer can obtain grades quickly and with good reproducibility.

DATA AND DISCUSSION

The effect of administering sodium fluoride and stannous fluoride in the drinking water at the same fluoride level to hamsters receiving a caries-producing diet is seen in Table I. The data indicate that in both sexes stannous

TABLE I

THE EFFECT ON DENTAL CARIES OF SUPPLEMENTING THE DRINKING WATER WITH SODIUM FLUORIDE AND STANNOUS FLUORIDE IN HAMSTERS RECEIVING A CARIOGENIC DIET

SUPPLEMENT	NUMBER OF ANIMALS	SEX	FLUORIDE CONCENTRATION OF THE DRINKING WATER (PPM)	WEIGHT GAIN DURING EXPERIMENTAL PERIOD (GM.)	MEAN S.I.	MEAN MA*	REDUCTION IN DENTAL CARIES (PER CENT)
Stannous fluoride	15	Male	10.6	63	7.07	5.4	53.3
	18	Female	10.6	65	7.25	6.0	49.1
Sodium fluoride	16	Male	10.4	61	11.21	8.1	26.0
	17	Female	10.4	70	10.44	7.6	26.7
None	14	Male	0.2	60	15.14	9.9	--
		Female	0.2	62	14.25	9.4	--

*Molars affected.

fluoride is about twice as effective as sodium fluoride in reducing the severity of dental decay in the molar teeth of hamsters. The application of "Student's"

T test to the S.I. data shows that the differences between sodium fluoride and stannous fluoride are highly significant. The probability that the difference shown by the males could be due to chance is only seven in ten thousand. The corresponding probability for the difference shown by the females is twelve in one thousand. These data corroborate and extend the previous findings using the albino rat.[1, 2] Fewer teeth were decayed in the animals receiving stannous fluoride than in those whose drinking water contained sodium fluoride. This is shown by the count of molars affected (MA), which is about two teeth less for stannous fluoride than for sodium fluoride.

Table II shows the analytic data for fluorine, calcium, and phosphorus in

TABLE II

FLUORINE, CALCIUM, AND PHOSPHORUS IN FEMURS OF HAMSTERS RECEIVING SODIUM FLUORIDE OR STANNOUS FLUORIDE AT LOW CONCENTRATIONS IN DRINKING WATER

SUPPLEMENT	SEX	DRY WEIGHT (GM.)	ASH WEIGHT (GM.)	ASH (%)	FLUORINE CONCENTRATION (PPM)	TOTAL F (MG.)	CALCIUM (MG. %)	PHOSPHORUS (MG. %)	Ca/P IN ASH
Stannous	M	0.183	0.097	49.7	1,378	0.125	39.2	19.7	1.99
fluoride	F	0.189	0.104	56.1	1,690	0.177	39.7	18.9	2.10
Sodium	M	0.184	0.094	50.7	1,795	0.167	38.2	18.6	2.05
fluoride	F	0.206	0.113	54.8	1,940	0.221	37.4	18.2	2.06
None	M	0.191	0.093	48.9	311	0.029	39.8	20.0	1.99
	F	0.193	0.101	54.0	326	0.032	39.5	19.6	2.01

the three groups of animals. Previous data[3] on the rat have indicated that stannous fluoride causes the storage of less fluorine in the skeletal system than sodium fluoride at the same stoichiometric equivalent of fluorine. The data reported in this article lend strength to this observation. Thus, in this test, the group of animals storing less fluorine had less caries. This suggests that the effectiveness of all fluorides in reducing the incidence of dental caries does not entirely parallel the uptake of such fluorides in the bones. This is at variance with the conclusion[4] that there is a parallelism between the uptake of fluorides in the bones and the effectiveness in preventing caries.

One conclusion that suggests itself is that the cation confers some specificity to the protective mechanism, and either is an adjunct to the fluorine ion itself or augments the effect of the fluoride ion by some additive mechanism. The caries reduction observed in hamsters which are started on the experimental procedures after calcification of the teeth must be a posteruptive effect; the marked effectiveness of stannous fluoride under these conditions suggests that it may be of unusual importance in reducing dental caries when applied topically.

SUMMARY

1. Stannous fluoride administered in the drinking water at a fluoride level of 10 ppm has been shown to be superior to a similar concentration of fluorine furnished by sodium fluoride in its ability to reduce the incidence of dental caries in the hamster. Both the severity of the caries attack and the number of teeth affected bear out this relationship.

2. Less fluorine is stored in the skeletal system when stannous fluoride is given in the drinking water than when an equal concentration of sodium fluoride is administered. No disturbance in calcification is noticed when either salt is used as judged by analysis of the femurs for calcium and phosphorus.

3. The reason for the superiority of stannous fluoride in reducing dental caries in the hamster is not understood.

4. A new method of evaluating the severity of dental caries in small animals is presented. It was developed in an attempt to provide a simple and adequate method for recording dental caries in the laboratory animal.

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THE INFLUENCE OF LATITUDE AND DISTANCE FROM SEACOAST ON DENTAL DISEASE

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THE purpose of this study is to bring together large geographical studies of dental disease among relatively homogeneous groups, plot them on maps, and apply statistical analysis to the plots. Such methods may lead to the recognition of etiological factors hitherto unstudied in their relation to dental disease. A number of reports have been made upon the geographical variations in dental disease found in military populations within the United States.[1-4] One study of children made by the United States Public Health Service[5] is large enough to give regional and often local comparisons. A few studies are available from other parts of the world showing variations in dental disease within widespread but racially somewhat homogeneous groups.[6-8] Although some efforts have been made by the authors of these studies and others[9, 10] to identify broad geographical factors which may be expected to influence dental disease anywhere in the world, much remains to be done in the plotting, analysis, and rationalization of the material which is available.

The term "dental disease" is used advisedly because, in lack of more accurate case histories, one cannot exclude teeth lost for reasons other than caries. Above the age of 40 or 50 years, tooth losses from periodontal disease may easily exceed losses from caries. Nevertheless, studies of decayed, missing, and filled teeth among children or young adults and of military acceptance based upon these conditions may be presumed to be predominantly studies of caries.

The difficulty of obtaining accurate dental data from large geographic areas is tremendous, even when populations are racially and socioeconomically relatively homogeneous. Existing nationwide surveys are far from ideal. Claude Bernard states appropriately that "we never compile statistics when we can possibly avoid it" and that "statistics deal with probability, never certainty." Nevertheless, probabilities are of interest when we are dealing with a disease which, like caries, seems to have multiple causation. The larger the population groups to be studied, the more unknown variables which are likely to be present, but also the greater the chance that these variables will even out. We must do the best we can with the material we have. Any patterns seen consistently in large studies should be taken seriously.

OBSERVATIONS

The United States.—Most of the large studies which lend themselves to analysis by states are of military populations. These populations, while not completely homogeneous, represent predominantly the white race and have in

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common certain cultural and physical denominators that are relatively reliable. Since recruiting and draft standards are national matters, each study group has some uniformity in age composition and physical excellence within itself, state by state. The nationwide transportation of many common food items tends toward a more or less uniform diet. East,[9, 15] Nizel and Bibby,[3] and others have noted the similarity of the various existing studies as to relative amounts of dental disease found in certain major regions of the country at intervals since the Civil War.

The three military studies best adapted to geographical analysis are those of Britten and Perrott,[1] of Ferguson,[2] and of Nizel and Bibby.[3] The first of these gives the prevalence of rejectable dental defects per 1,000 drafted men among men who were rejected or accepted for limited duty in the first World War. Rates range from 2.90 to 102.85 per 1,000. They are based on examinations of 967,486 men at camps after May 1, 1918, when examinations had become "more efficient and standardized." The second study gives the average number of decayed, missing, and filled teeth per recruit among 4,602 white Naval recruits seen at one induction center in peacetime. The third study gives average numbers of decayed and missing teeth per selectee among 22,117 men at a large Army camp in World War II. Each study has defects from a statistician's point of view. Even a comparison of decayed and missing teeth between the two studies which have these measures in common would be out of the question. Internal comparisons, however, are probably justifiable on the basis of a rank list of states from 1, the state with the lowest prevalence of dental disease, to 48, the state with the highest.

The 1918 study, from records sent in from all over the country, has the advantage that a very simple measure is involved: acceptance or rejection according to numbers of teeth "present and serviceable" in the mouth. The other studies were each made at one station, with some chance for uniformity in examination method.

Table I gives the three rank lists made by averaging the three figures for each state. Each study in itself shows a pronounced geographical pattern for the prevalence of dental disease similar to the other in major outline, but with occasional discrepancies. The act of combining the rank lists seems to preserve the pattern but iron out many of the discrepancies. Note for instance the unusual rank for Delaware (forty-eighth) among Atlantic Coast States in the Army study. This rank, based on a very small sample, is not as realistic as one which represents the combined samples of all three studies.

Correlations can be made between the three rank lists by Spearman's method.* A coefficient of correlation results which may vary from 1.0 (complete positive correlation) to −1.0 (complete negative correlation) just as does a coefficient by the usual formula. A coefficient at or near 0 expresses lack of relationship. The study of draftees in 1918 correlates with the Army

*The formulas used were:

$r^1 = 1 - \frac{6\Sigma\Delta^2}{n(n^2 - 1)}$ where Δ is the difference between ranks in a pair of observations and n the number of pairs in the series. (Σ means "sum of.")

TABLE I

RANKS OF STATES IN PREVALENCE OF DENTAL DISEASE; THREE LARGE MILITARY STUDIES SEPARATELY AND COMBINED¶

STATE	DRAFTEES 1918*	NAVY 1934†	ARMY 1943‡	MEAN RANK
Alabama	11	5.5§	27	14
Arizona	8	24.5‖	12	15
Arkansas	1	1	5	2
California	19	24.5	14	19
Colorado	13	24.5	9	15
Connecticut	41	48	35	41
Delaware	40	37.5	48	42
Florida	34	7	10	17
Georgia	30	3.5	23	19
Idaho	22	24.5	38	28
Illinois	24	36	29	30
Indiana	12	16	30	19
Iowa	15	14	20	16
Kansas	2	10.5	6	6
Kentucky	9	15	24	16
Louisiana	36	10.5	18	21
Maine	46	40	47	44
Maryland	31	37.5	34	34
Massachusetts	44	47	41	44
Michigan	39	33	31	34
Minnesota	28	35	26	30
Mississippi	17	3.5	13	11
Missouri	6	17	28	17
Montana	35	24.5	11	23
Nebraska	5	31	17	18
Nevada	18	24.5	7	16
New Hampshire	47	40	42	43
New Jersey	43	42	43	43
New Mexico	4	24.5	4	11
New York	42	44	45	44
North Carolina	23	5.5	16	15
North Dakota	27	24.5	32	28
Ohio	20	43	39	34
Oklahoma	7	10.5	3	7
Oregon	37	24.5	44	35
Pennsylvania	38	46	33	39
Rhode Island	45	45	46	45
South Carolina	26	10.5	19	18
South Dakota	29	18	21	23
Tennessee	14	2	15	10
Texas	3	10.5	1	5
Utah	25	24.5	22	24
Vermont	48	40	36	41
Virginia	32	10.5	8	17
Washington	33	24.5	37	31
West Virginia	16	34	25	25
Wisconsin	21	32	40	31
Wyoming	10	24.5	2	12

*Britten and Perrott.[1] Rejection rates for dental defects.

†Ferguson.[2] DMF per person.

‡Nizel and Bibby.[3] Decayed and missing teeth per person.

§Where more than one state had the same DMF count the ranks of the states were averaged.

‖All western states ranked 24.5 were in a pooled sample.

¶Low rank implies low prevalence.

study in 1943 by a coefficient of 0.708. The Navy study in 1934 correlates with the draftee study by a coefficient of 0.596 and with the Army study by a coefficient of 0.665. These last two poorer correlations are probably due to the fact that twelve western states have been pooled into a single sample in the

Navy study and can be given only one rank. Even the first coefficient (0.708) is not high for two studies which attempt to measure the same thing. Inaccuracies in method, the long time interval between the studies and unknown factors all may contribute to this result.

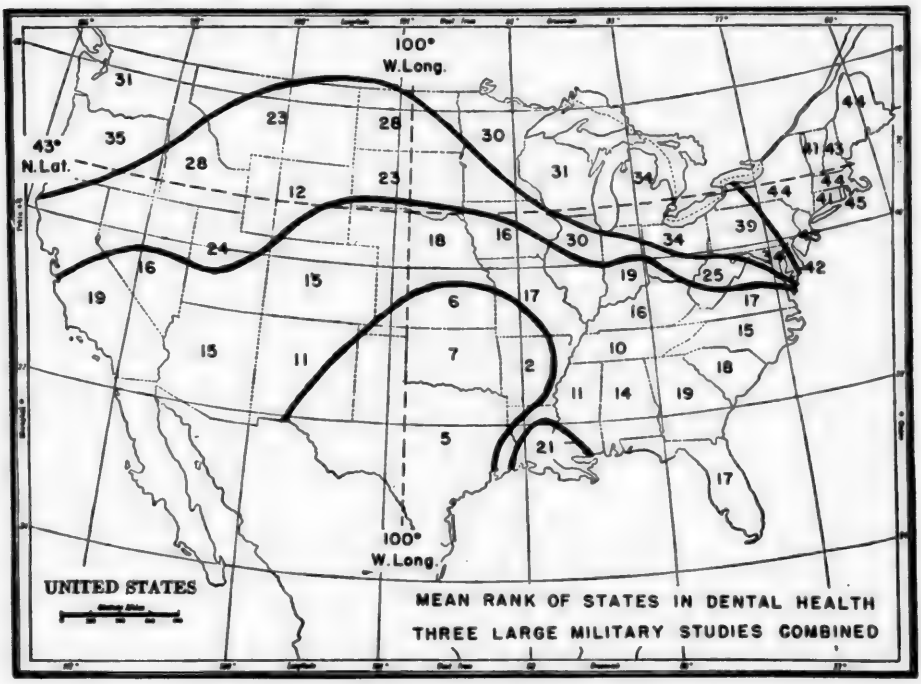


Fig. 1.

The mean ranks for states are plotted in Fig. 1. The list contains no Number 1 and no Number 48, since no state was at the beginning or end of the list in all three studies. Lines have been drawn to group the ranks by tens as far as possible. It is of interest that any such lines can be drawn without gross overlapping. The pattern of prevalence of dental disease which emerges suggests two striking associations: one, latitude, the other, distance from seacoast. It seems impossible to interpret the map without considering both of these factors. Statistical testing of the correlation between each of the factors and the prevalence of dental disease is possible, as shown in Tables II, III, and IV.*

*The formulas used were:

$$r = \frac{\frac{\Sigma f x_1 x_2}{n} - \bar{m}_1 \bar{m}_2}{\sigma_{obs_1} \sigma_{obs_2}} \quad \text{and} \quad \sigma_{obs} = \sqrt{\frac{\Sigma f x^2}{n} - \bar{m}^2}$$

where x_1 and x_2 are the scores and $\bar{m}_1$ and $\bar{m}_2$ the means in the two series of observations. σ_{obs} is the standard deviation of the observations. Σ means "sum of" and f means frequency.

TABLE II

APPROXIMATE LATITUDE OF CERTAIN STATES ON THE ATLANTIC COAST, COMPARED WITH THEIR MEAN RANKS IN THREE STUDIES OF DENTAL DISEASE

STATE	RANK	LATITUDE (DEGREES N.)
Florida	17	28
Georgia	19	33
South Carolina	18	34
North Carolina	15	36
Virginia	17	38
Maryland	34	39
Delaware	42	39
New Jersey	43	40
New York	44	42
Connecticut	41	41
Rhode Island	45	42
Massachusetts	44	42
New Hampshire	43	43
Maine	44	44

Correlation coefficient, 0.844. Probability of chance occurrence, less than 1 in 1,000.

Table II shows a latitude correlation for states along the Atlantic Coast, arranged in order of latitude but not necessarily all on the same meridian of longitude. The object in selecting this group of states was to reduce as nearly as possible to zero the influence of distance from seacoast. There is obvious difficulty in computing the geographical center of population for each state. New York, particularly, combines large seacoast and large inshore population groups, and here, too, we find considerable variations in latitude within the state. Most other states are easier to interpret. The correlation coefficient for states in Table II is 0.844. If Fisher and Yates' confidence intervals[11] are used, there appears a probability of less than 1 in 1,000 that this coefficient could be the result of change. The correlation is therefore interpreted to be highly significant. "Significance" here must be understood to apply specifically to the mathematical part of the analysis. The reader, to make his own over-all estimate of significance, must combine mathematical considerations with a common-sense appraisal of the accuracy, grouping, etc., of the original data.

TABLE III

APPROXIMATE LATITUDE OF CERTAIN STATES ON THE ONE HUNDREDTH MERIDIAN OF WEST LONGITUDE, COMPARED WITH THEIR MEAN RANKS IN THREE LARGE STUDIES OF DENTAL DISEASE

STATE	RANK	LATITUDE (DEGREES N.)
Texas	5	32
Oklahoma	7	35
Kansas	6	39
Nebraska	18	41
South Dakota	23	44
North Dakota	28	47

Correlation coefficient, 0.923. Probability of chance occurrence is just less than 1 in 100.

Next, it is of interest to compare a group of inshore states of different latitude. Such a group is shown in Table III. The states along the one hundredth meridian of west longitude were chosen in order to avoid so far as possible the presumed influence of the Gulf of Mexico. (Note the high prevalence

of dental disease in Louisiana.) It is desirable to avoid the states farther west where areas are greater and populations are comparatively smaller. The states from Texas to North Dakota again show an almost perfect progression in prevalence of dental disease. The correlation coefficient between mean rank and latitude is 0.923: a strikingly high coefficient, but not quite as significant as that in Table II because of the small number of states involved. Probability of chance occurrence is just less than 1 in 100.

Corroboration of this increase in dental disease with latitude is found among the children reported in *Public Health Bulletin No. 226*. Mills[10] has analyzed the caries experience of the rural white boys 12 to 14 years of age in this study group. With data from only nineteen states he found evidence of higher caries experience in northern latitudes. *Bulletin No. 226,* based as it is on questionnaire reports, must be used with caution, but internal comparisons there can be accepted as of some value. Large samples are available, a fact which tends to offset the usual high variability in dental caries experience among individuals.

TABLE IV

APPROXIMATE DISTANCE FROM SEACOAST OF CERTAIN STATES ON THE FORTY-THIRD PARALLEL OF LATITUDE, COMPARED WITH THEIR MEAN RANKS IN THREE LARGE STUDIES OF DENTAL DISEASE

STATE	RANK	DISTANCE FROM SEACOAST* (MILES)
New Hampshire	43	50
Vermont	41	150
New York	44	100
Michigan	34	550
Wisconsin	31	800
Iowa	16	950
South Dakota	23	1,300
Nebraska	18	1,100
Wyoming	12	850
Idaho	28	600
Oregon	35	100

Correlation coefficient, −0.847. Probability of chance occurrence approximates 1 in 1,000.
*Distance in miles from approximate center of population to nearest seacoast.

The relationship of dental disease to distance from seacoast in the military groups can be studied on almost any parallel of latitude. The forty-third parallel has been chosen, since eleven states abut on or are crossed by this parallel. An estimate of the distance of the approximate center of population of each state from the nearest salt water has been made and correlated with the mean rank of the state for dental disease. Table IV shows the result. A correlation coefficient of −0.874 results. This coefficient is highly significant, since the probability of its occurring by chance is approximately 1 in 1,000. Mills again foreshadowed this finding when he stated from an analysis of four river valleys that "caries (tends) to increase as one passes from the headwaters of a river toward its mouth."

South Africa.—Ockerse[6] has published a vast amount of data from the Southern Hemisphere which can be analyzed in a manner similar to that used

in the United States. His map of percentages of children with dental caries in the magisterial districts of the Union of South Africa gives a pattern which is almost the inverted image of that seen in the United States. Fig. 2 shows a plotting of these figures, with magisterial districts having more than 0.70 parts per million of fluoride in the water supply excluded in order to minimize the known influence of fluoride. Those districts with 0.50 to 0.69 ppm have been circled. It is of interest that the pattern seen in the mapping of figures for all magisterial districts remains unaltered.

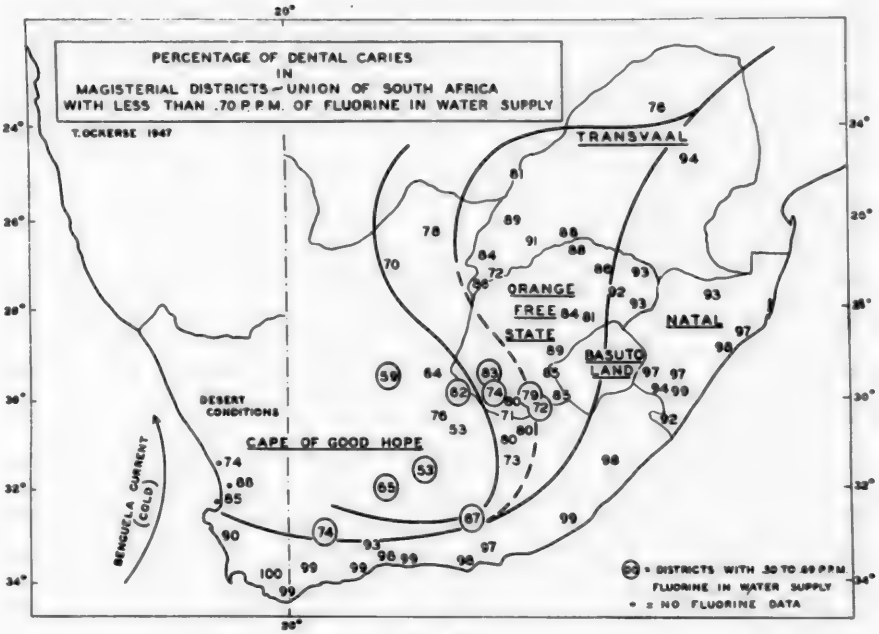


Fig. 2.

The shape of the Union of South Africa makes it difficult to separate the influence of latitude from that of distance from seacoast. For convenience, the latter has been used in statistical analysis. The area covered by Ockerse's data can be divided into zones of 100 miles each from the nearest seacoast. Table V shows the effect of this zoning. The correlation coefficient which results from analysis of the table is −0.398. This coefficient, though small, is based on a large number of observations and is considered statistically significant. The probability that such a relationship could have occurred by chance is between 1 in 100 and 1 in 1,000. It is of interest that this correlation is based almost entirely on fifteen seacoast districts which show 95 to 100 per cent caries. Inshore, there is little pattern to the data.

For 150 miles north of Capetown seacoast districts show higher caries than those in land, but dental data from the sparsely settled Northwest Cape area beyond show low caries prevalence right to the coast. Here, then, is

an exception to the seacoast relationship seen elsewhere. An apparent explanation lies in the fact that northwest of Capetown the cold Benguela Current sweeps in from the Antarctic Ocean to produce desert conditions, just as does the Humboldt Current in Chile. Further study of specific etiological factors which vary with distance from seacoast would be of interest in the Northwest Cape area: particularly relative humidity and total water hardness, both to be discussed later.

TABLE V

APPROXIMATE DISTANCE FROM SEACOAST OF CERTAIN MAGISTERIAL DISTRICTS,* UNION OF SOUTH AFRICA, COMPARED WITH PERCENTAGE OF CARIES IN THOSE DISTRICTS (OCKERSE, 1949)

PERCENTAGE CARIES	DISTANCE FROM SEACOAST IN MILES					ALL DISTANCES
	0 TO 99	100 TO 199	200 TO 299	300 TO 399	400 TO 500	
50- 54		2				2
55- 59			1			1
60- 64			2			2
65- 69	1	1				2
70- 74	1	3	1	1	1	7
75- 79		1	2		1	4
80- 84		3	4	2	1	10
85- 89		2	2	2		6
90- 94	3	2	2	1		8
95-100	15					15
All percentages	20	14	14	6	3	57

Correlation coefficient, −0.398. Probability of chance occurrence is between 1 in 100 and 1 in 1,000.

*All districts east of 20° east longitude excepting those having 0.7 ppm or more fluoride in the water supply and those not recorded.

Ockerse has published some interesting coefficients of his own and finds caries significantly associated with fluoride in the water supply, water hardness, and hydrogen ion content of water. Fluoride and water hardness he also finds to be associated with each other. Further analysis of his data shows that fluoride is not significantly correlated with distance from seacoast in the Union of South Africa, but that water hardness is so correlated. The coefficient for water hardness and distance from seacoast is 0.462 (significant).

TABLE VI

APPROXIMATE LATITUDE OF CERTAIN STATES IN AUSTRALIA COMPARED WITH MEAN DMF TOOTH COUNTS IN AIR FORCE PERSONNEL DRAWN FROM THESE STATES (ANDREWS, 1948)

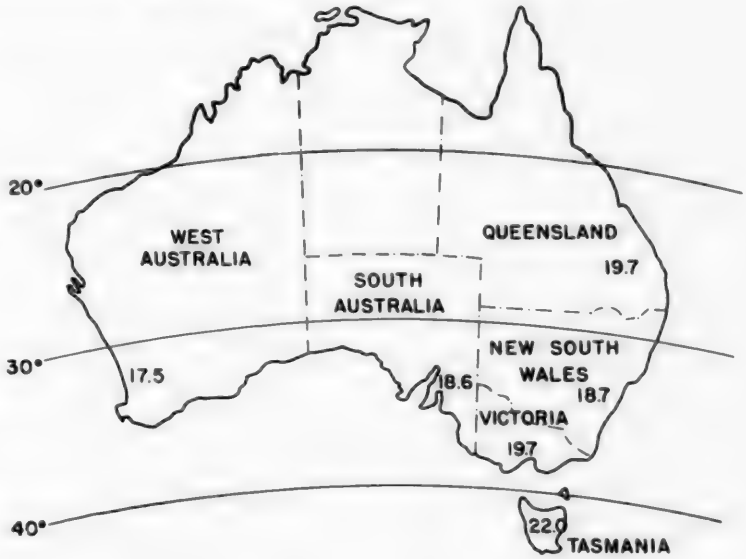
STATE	DMF TEETH	LATITUDE* (DEGREES S.)
West Australia	17.50	32
South Australia	18.57	34
New South Wales	18.66	33
Queensland	19.72	25
Victoria	19.74	37
Tasmania	21.98	42

Correlation coefficient, 0.540. Probability of chance occurrence is greater than 1 in 10.

*Approximate latitude of the center of population of the state.

Australia.—Andrews[7] studied 2,000 members of the Royal Australian Air Force, dividing his data by states. He found that counts of DMF (decayed, missing, and filled) teeth vary as shown in Fig. 3. A relation to latitude is

apparent as shown in Table VI. Analysis shows a correlation coefficient of 0.540. This coefficient is not statistically significant, as is to be expected where observations are grouped into a small number of categories. Table III shows a significant result only because the correlation coefficient is exceptionally high. It is of equal interest, therefore, to consider how the mean values for individual states were developed and what significance can be attached to differences between these means.



DMF TEETH AMONG AUSTRALIAN AIR FORCE PERSONNEL 1939—1945

Fig. 3.

Large differences are often significant, even though the samples producing the means are not very large. Smaller differences are significant only if samples are large enough and standard deviations not too large. Andrews did not give the size of his state samples, but mentioned that his 2,000 cases were assembled by a random sampling method. Each state sample might therefore approximate 330 cases. Dunning[12] has shown that human DMF counts vary according to a somewhat regular pattern, with the standard deviation of the observations often falling between 4 and 5 teeth per person. If we assume, conservatively, a sample size of 300 and a standard deviation of 5, it seems likely that differences of more than about 1.3 teeth between means will show statistical significance. There are actually seven differences in the Andrews material greater than 1.3: West Australia differs from Queensland and Victoria by more than this amount, and Tasmania differs similarly from all the other states. It seems reasonable, therefore, to attach some significance to the major geographical pattern seen here for Australia.

No reliable correlation between dental disease and distance from seacoast can be made in Australia, since most of the large population centers are near the seacoast and there are no interior states.

The *1951 Yearbook for the Commonwealth of Australia*[13] gives interesting climatological data for the Australian capital cities which can be compared with the Andrews figures for dental disease. Table VII gives the data and

TABLE VII

RANKS OF AUSTRALIAN STATES IN DENTAL DISEASE COMPARED WITH RANKS OF THEIR CAPITAL CITIES IN CERTAIN CLIMATOLOGIC FEATURES

STATE AND CITY	DENTAL DISEASE		MEAN DAILY SUNSHINE		MEAN TEMPERATURE		MEAN RELATIVE HUMIDITY	
	DMF TEETH	RANK	HOURS	RANK	DEGREES F.	RANK	PER CENT	RANK
West Australia—Perth	17.50	1	7.8	6	64.5	5	62	2
South Australia—Adelaide	18.57	2	7.0	4	63.1	3	52	1
New S. Wales—Sydney	18.66	3	6.8	3	63.7	4	68	4
Queensland—Brisbane	19.72	4	7.5	5	69.0	6	67	3
Victoria—Melbourne	19.74	5	5.6	1	58.8	2	69	6
Tasmania—Hobart	21.98	6	5.8	2	54.4	1	65	5

Rank correlations (Spearman): Dental disease—sunshine $r' = -.771$. Dental disease—temperature $r' = -.600$. Dental disease—humidity $r' = 0.829$.

the corresponding rank lists. It is interesting to note that mean annual hours of sunshine, temperature, and relative humidity all vary more or less together, the first two rising as the third declines, and that all three are related fairly well to the variations in dental disease. Dental disease correlates best with relative humidity ($r' = 0.829$) and also fairly well with sunshine ($r' = -0.771$). Because of the small number of observations, only the coefficient for relative humidity approaches the 5 per cent level of significance.

New Zealand.—Saunders has published a monograph[8] describing in detail the major variations of dental caries in New Zealand. His material, when plotted, does not correlate with latitude, perhaps because of the small size of the country. He makes various analyses of etiologic factors and is able to draw conclusions from the socioeconomic differences between his sources of data. Farming communities, for instance, show low caries, and industrial and coal mining communities show high caries. A strong correlation exists between caries in children and supervision of the Plunket nurses who administer a carefully planned system of nutrition for expectant mothers and infants. The differences in latitude in New Zealand, if they exert any influence at all, are not large enough to overcome these stronger local factors.

INTERPRETATIONS

Etiology of Latitude Variations.—The identification of variations in dental disease with latitude and distance from seacoast will prove most useful if it helps us in a study of individual etiological factors for caries or for periodontal disease. The largest group of such factors is climatological and includes sunshine, rainfall, humidity, and temperature. These factors are often

MEAN ANNUAL SUNSHINE, U.S. WEATHER BUREAU MAP No. 13

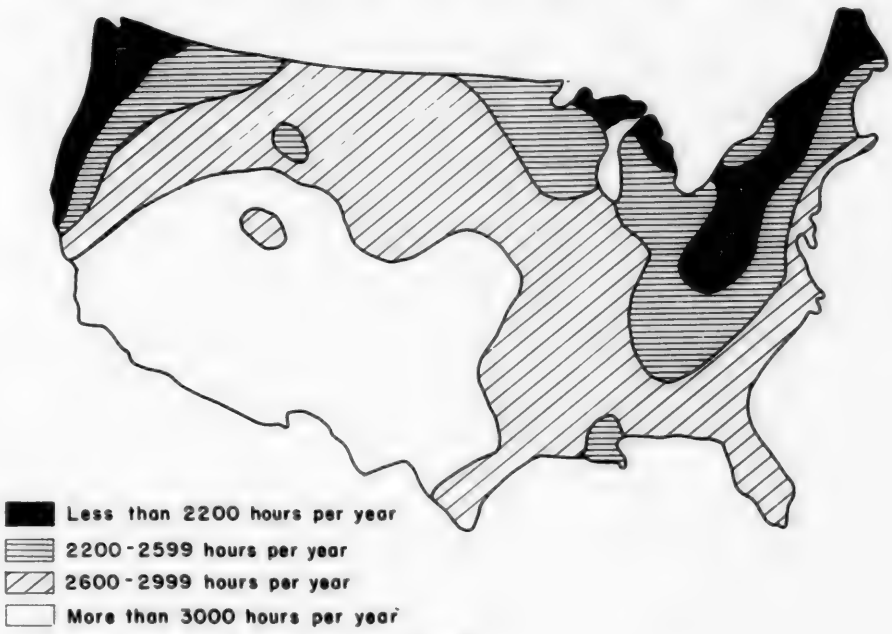
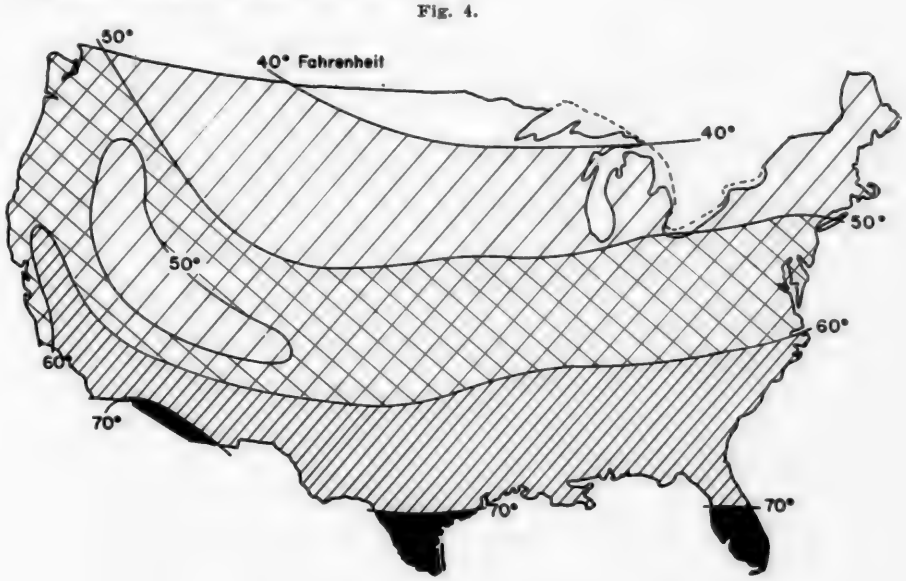


Fig. 4.



MEAN ANNUAL TEMPERATURE, U.S. WEATHER BUREAU BULLETIN S

Fig. 5.

related to one another, directly or inversely, but they deserve separate study. Chemical composition of water supply and urbanization are other important factors. Any effort to divide all these factors into those associated either with latitude or with distance from seacoast is somewhat arbitrary, for a factor such as sunshine may often be related to both. Such an effort, however, is a necessary part of the analysis of the obvious geographic trends in dental disease and will help us to an understanding of the factors themselves.

Sunshine is one of the factors most commonly thought to vary with latitude. The measurement of sunshine is not a simple matter. Total possible hours of sunshine per year actually increase a little as one leaves the equator,[14] since long days in summer compensate for long nights in winter. Actual hours of sunshine in the absence of cloud cover give a better measure and one which generally decreases as one leaves the equator. Even this does not tell as much as it might about sunshine available to human beings, for a given ground area intercepts less sunlight when the sun is low in the sky, temperature falls, and human beings clothe themselves or are driven indoors. The atmosphere, too, intercepts more ultraviolet light when the sun is low.

Mean annual hours of actual sunshine are shown on United States Weather Bureau Map No. 13 from which Fig. 4 has been adapted. This map shows a pattern much similar to that for dental disease in the United States (Fig. 1), at least in terms of latitude variation. East[15] compared dental caries among the rural children reported in *Public Health Bulletin No. 226* with the mean annual sunshine where they live and found an inverse relation-

TABLE VIII

MEAN DAILY HOURS OF SUNSHINE IN CERTAIN AREAS IN THE UNION OF SOUTH AFRICA COMPARED WITH DENTAL DISEASE IN THE SAME AREAS

	MEAN DAILY SUNSHINE		PER CENT CARIES	
	HOURS	RANK	PER CENT	RANK
Inland Areas				
Johannesburg	8.70	6	97	5
Beaufort West	9.21	7	82 (65*)	1
Victoria West	9.73	9	75 (74*)	2
Kimberly	9.40	8	94	3
Coastal Areas				
Cape Town	7.56	5	98	6.5
Mossel Bay	7.30	3	98 (99*)	8
Port Elizabeth	7.55	4	99	9
Nelspruit	7.20	2	95	4
Durban	5.80	1	98	6.5

Rank correlation (Spearman): $r' = -.879$.

*Percentage of caries in the adjoining district. The two figures are averaged in computing rank.

ship which is highly significant statistically. Ockerse gave sunshine figures for certain areas in South Africa. When these figures are compared with those for dental disease in the same areas (Table VIII) a high rank correlation of −0.879 results. The reasons for this relationship need further study, particularly the analysis of ultraviolet light reaching the earth at various places.

Temperature varies almost entirely with latitude, as is shown in Fig. 5. The only other factor to vary temperature seems to be high altitude, as is

seen in the Rocky Mountain area. Temperature, in turn, acts to vary the caloric requirements and water intake of human beings. Carbohydrate food is not only a quick, but a relatively cheap, source of caloric energy. Our knowledge of the etiology of caries, therefore, indicates a way in which this disease may be related to temperature. Data on variations of civilized diet with latitude or temperature is scarce. One careful study by the United States Department of Agriculture[16] shows the consumption of baked goods (breads, cakes, and pastries not baked at home) to be sharply higher in the north. Table IX gives the figures. Consumption of sugar was found to be

TABLE IX

AVERAGE QUANTITIES OF BAKED GOODS* CONSUMED AT HOME IN ONE WEEK, FIVE ANALYSIS UNITS IN TWENTY STATES, MARCH TO NOVEMBER, 1936; NONRELIEF FAMILIES THAT INCLUDE HUSBAND AND WIFE, BOTH NATIVE-BORN (STIEBELING, AND OTHERS[16])

REGION	CONSUMPTION IN POUNDS		
	FARMS	VILLAGES	SMALL CITIES
New England, Middle Atlantic, and North Central	7.2	8.4	7.8
Plains, Mountain and Pacific	4.3	6.7	7.3
Southeast: white operators	1.4	4.1	4.9
white sharecroppers	.8		
Negroes	.4	1.2	1.6

*Includes breads, cakes, and pastries not baked at home.

higher among northern farm families than among farm families elsewhere in the country. Variations in sugar consumption among families of other types and of syrups (molasses, jams, jellies, and candies) among families of all types were not striking. Further studies of carbohydrate consumption are needed, as well as further basic studies on carbohydrate nutrition and on methods of "refinement."

Urbanization. Ferguson[2] attributed the regional variations he found in caries to the greater frequency of large industrial cities in the North. This claim deserves examination, and *Public Health Bulletin No. 226* affords dental data on a number of cities, together with similar data on nearby county balances taken from communities of less than 5,000 population. The defects of *Bulletin No. 226* have been mentioned. Nevertheless, errors should be at a minimum when comparing closely adjoining samples within it, and no other useful material seems available. Forty-one cities were found of populations over 25,000, each of which could be matched by a rural sample. Most of the rural samples were taken from adjoining counties in order to avoid city suburbs, but a few samples were taken from the same county where the city was not over 100,000 population and the county was large. Similarity of communal water supply cannot be assured, but care was taken to select areas of similar distance from seacoast, lake, or major river. Table X shows the frequency of different caries scores for white boys 12 to 14 years of age in the areas involved. Urbanization is accompanied by only an 11 per cent increase in caries. Twenty-eight of the sample pairs show higher caries in the cities, thirteen show higher caries in the rural areas. Variation is so great that a

TABLE X

FREQUENCY OF CERTAIN AMOUNTS OF DENTAL CARIES IN WHITE BOYS 12 TO 14 YEARS OF AGE IN FORTY-ONE UNITED STATES CITIES OF OVER 25,000 POPULATION AND FORTY-ONE AREAS IN ADJOINING COUNTIES OF NOT OVER 5,000 POPULATION, 1933-34 (PUBLIC HEALTH BULLETIN NO. 226)

DMF TEETH PER 100 BOYS*	CITIES†	ADJOINING RURAL AREAS‡
150-199	–	2
200-249	3	4
250-299	3	5
300-349	2	6
350-399	5	3
400-449	8	2
450-499	7	8
500-549	2	4
550-599	4	4
600-649	3	2
650-699	2	–
700-749	1	–
800-849	1	–
950-999	–	1
	41	41
Means	460.5 teeth per 100	414.0 teeth per 100

*Permanent teeth per 100 boys carious, indicated for extraction, extracted, and filled.

†The cities were: Elkhart, Kokomo, Gary, Michigan City, Indianapolis, East Chicago, Hammond, South Bend, Mishawaka, Lafayette, Evansville, Terre Haute, and Richmond, all in Ind.; Des Moines, Iowa; Flint, Bay City, Kalamazoo, Muskegon, and Port Huron, Mich.; Minneapolis, St. Paul, and Duluth, Minn.; Lincoln and Omaha, Neb.; Camden, Atlantic City, and Trenton, N. J.; Portsmouth, Ohio; Johnstown, Reading, Lancaster, and Wilkes-Barre, Pa.; Sioux Falls, S. D.; Nashville, Memphis, Chattanooga, and Johnson City, Tenn.; Salt Lake City and Ogden, Utah; Richmond, Va.; and Sheboygan, Wis.

‡Each city is matched by a sample from areas of not over 5,000 population in an adjoining county of similar distance from seacoast. Occasionally the rural sample is taken from the same county, where the county is large and the city not over 100,000 in population.

significance test shows a critical ratio of only 1.43. A difference such as was found is therefore well within the range of chance occurrence.

A smaller, but probably more accurate, study of urbanization is found in Fulton's report on the New Zealand national dental service.[17] A sample of 4,072 children was selected to include 2,048 rural and 2,024 urban children, all ages from 7 to 14 being equally represented. Small differences are found in DMF teeth, the younger rural children (7 through 12) and the older urban children (13 and 14) showing the highest counts. Fulton stated that "neither these differences in average DMF nor any other differences between corresponding figures . . . seem to be significant. The striking fact is that the rural children appear to have received quite as good care as the urban children."

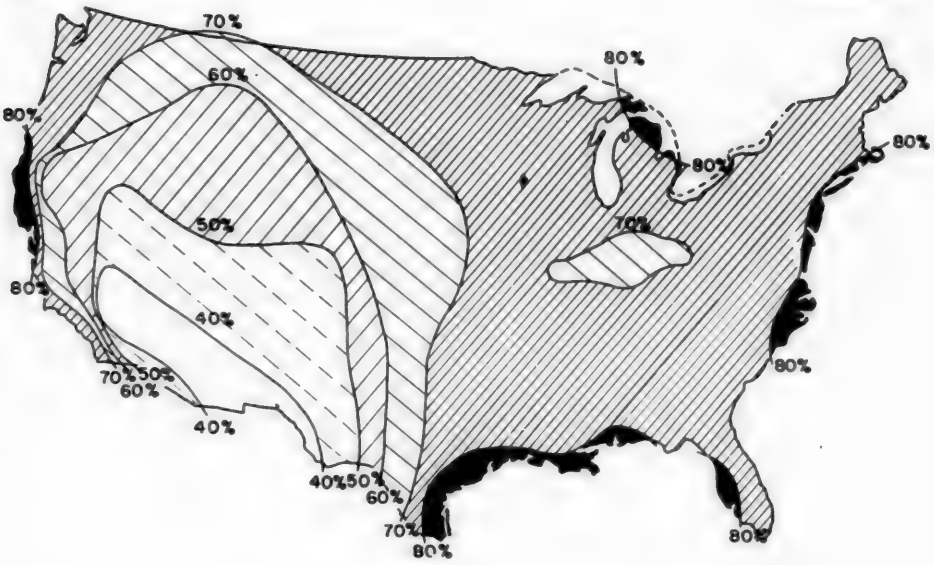
Etiology of Variations With Distance From Seacoast.—Fluorine, because of recent research, is the etiologic factor one would first attempt to relate to geographical variations in dental caries. Van Burkalow's mapping[18] of maximum fluoride concentrations in communal and noncommunal water supplies on a county basis gives the most comprehensive material available for the United States. No latitude variation is evident. Area for area, there are as many counties with fluoride maxima above 1.5 parts per million in the Dakotas as there are in Texas, and a similar situation holds to the east and to the west. Van Burkalow discounts climatic variations and feels that differences in geologic formation are probably more important. There is, however, some relation between fluoride maxima and distance from seacoast. The coastal states seem to have somewhat fewer high fluoride maxima, though un-

mapped areas in these states contribute to the impression. Correlation between fluoride and distance from seacoast is probably much better than is shown on Van Burkalow's map, for one deep-well water supply in a county may provide a high maximum figure, while the bulk of the county uses surface supplies (river, lake, or shallow well) which are almost always fluoride-free. Rivers are larger near the seacoast, and surface water is more commonly used there than it is inshore. This reasoning may apply to South Africa, too, where correlation between fluoride and distance from seacoast is lacking, as previously noted.

There is growing evidence that not all the variation in caries with distance from seacoast can be laid to fluoride. Hadjimarkos[19] found marked caries differences between counties on the Oregon seacoast and others not far away, but at higher altitudes and back of the Cascade Range. In this study none of the communities had appreciable fluoride in the water supply. Exclusion of high-fluoride communities from Ockerse's data (Fig. 2 and Table V) leaves a convincing correlation between caries and distance from seacoast.

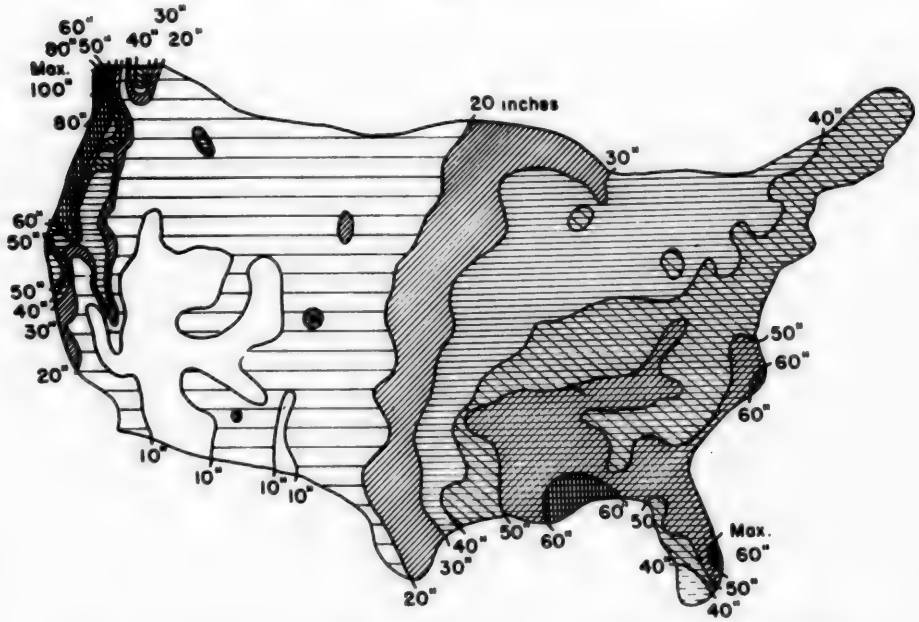
Total water hardness, usually measured in terms of calcium carbonate is an etiological factor in caries that has been known for many years. More recently it has faded from sight as attention was focused more sharply on fluorides. Röse,[20] Mills,[10] and Ockerse[6] are among the authors who have reported an inverse relationship. Ockerse's correlation coefficient between calcium carbonate and percentage of caries is −0.811, even higher than his coefficient of −0.662 between caries and fluorides. If his data for calcium carbonate are plotted on a map zoned in hundreds of miles from seacoast, a significant correlation coefficient of 0.462 is obtained for the increase seen as one proceeds inland. All of this returns our attention sharply to total hardness. Known components of hardness deserve further study. Trace elements must be studied and factors now unknown must be sought.

Relative humidity is a measure of the amount of moisture in the atmosphere in proportion to the amount to be tolerated without precipitation at a given temperature and barometric pressure. It is a better indication of the dampness of a climate than is actual precipitation. A mapping of the mean annual relative humidities over a period of years in the United States (Fig. 6) shows these humidities to be greatest along the immediate seacoasts, both Atlantic and Pacific. From a short distance inshore, east of the Mississippi, most of the area shows little variation from percentages in the middle 70's (the "humid East"). The western states show sharp and regular variations with both distance from seacoast and latitude, giving an appearance to the map much like that for dental caries. If the data for individual weather stations in *United States Weather Bureau Bulletin O* are combined to give state averages and these values are arranged in order of rank, a correlation can be made with the mean ranks for dental disease in Table I. This process gives a significant coefficient (r', Spearman) of 0.548. Reference to Fig. 6, however, shows that many sharp changes in relative humidity cut across states, especially those on the seacoast. A better study can be done.



MEAN ANNUAL RELATIVE HUMIDITY, U.S. WEATHER BUREAU BULLETIN O

Fig. 6.



MEAN ANNUAL PRECIPITATION, U.S WEATHER BUREAU BULLETIN D

Fig. 7.

The data from Australian states (Table VII) shows a higher correlation (0.829) between caries and relative humidity than between caries and any other climatic factor. Relative humidity, for these reasons, needs careful study as a factor in the etiology of caries.

Rainfall, leaching minerals from the soil and blocking sunlight, is another factor to be considered. Fig. 7 shows mean annual precipitation for the United States. Though no latitude relationship is evident, there is a regular decrease in rainfall as one proceeds inshore. Only on the Atlantic coast is this pattern at variance with the one for prevalence of dental disease. Rainfall, though decreasing inland, is greater in the South than in the North. The mechanisms by which relative humidity and rainfall might be linked to dental caries, either together or separately, need further study. Nizel and Bibby[3] found disappointing results when they attempted to relate soil composition analyses to dental caries.

Sunshine, though related to latitude, is not consistently related to distance from seacoast. Sunshine increases as one proceeds inshore from the Pacific Coast, decreases as one proceeds inshore from the Atlantic. In South Africa, it increases as one proceeds inshore. *Urbanization* might be a factor insofar as many large cities are located on the seacoast. For reasons already stated, urbanization is likely to prove unimportant.

SUMMARY

A number of large studies of dental disease in the United States, South Africa, Australia, and New Zealand are analyzed graphically and statistically. While none of these studies offer all one could wish in either quality or quantity, they merit our best efforts at interpretation, if only to stimulate more and better work in the future.

A suggestive pattern emerges for the United States showing dental disease (chiefly caries) to increase with latitude and with proximity to seacoast. Both of these relationships are verified by calculations which show statistically significant correlation coefficients. In observations from the Southern Hemisphere a similar pattern, inverted, is found in South Africa. This, too, is statistically significant. Variation with latitude is apparent in Australia.

A group of climatological factors, together with the chemical composition of water supplies, probably account for most of the variations in dental disease. The climatological factors are sunshine, rainfall, temperature, and relative humidity—all more or less related to one another. The relation of sunshine to dental disease is confirmed in this study, that of relative humidity demonstrated.

The major factors involved in variation with latitude appear to be sunshine and temperature. Variation in mean annual hours of sunshine present a pattern much like that for dental disease in the United States, with considerable latitude changes. Statistical correlations are significant. Temperature varies almost entirely with latitude and may be linked to dental

caries through its effect on the caloric requirements and water intake of humans. The consumption of carbohydrate food seems to be greater in northern latitudes. Urbanization has been claimed as a reason for higher dental caries in the north. Data from forty-one cities in the United States show caries to be slightly, but not significantly, higher in these cities than in adjoining rural areas.

The major factors involved in variations with distance from seacoast appear to be fluorides, total water hardness, and relative humidity. Fluoride is most common in deep-well waters, and deep wells are most common in inshore areas. The persistence of caries variation in South Africa, even after the removal of high fluoride communities there, returns our attention to water hardness and to climatological factors. Relative humidity in the United States presents a pattern much like that for dental disease except that a "plateau" exists in the eastern states from just back of the immediate seacoast to the Mississippi Valley, with none of the variations seen for dental disease in this area. Sunshine varies somewhat with distance from seacoast, but decreases as one proceeds inshore from the Atlantic Coast. Rainfall decreases as one proceeds inshore from either ocean, but does not present a geographical pattern like that for dental disease.

It is hoped that identification of major geographic trends in the prevalence of dental disease will permit a more systematic approach to the epidemiology of dental caries. This may lead to productive new studies on specific etiological factors and may provide valuable clues to more basic research. At the very least, it can provide the basis for estimating treatment needs in different areas.

Grateful acknowledgments are due to Hugo Muench, Professor of Biostatistics, and Jane Worcester, Associate Professor of Biostatistics, Harvard School of Public Health, for assistance in statistical method. Valuable Criticism was also received from Reidar F. Sognnaes, Brackett Professor of Oral Pathology, Harvard School of Dental Medicine, and Basil G. Bibby, Director, Eastman Dental Dispensary, Rochester, N. Y.

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CARIESLIKE ENAMEL CHANGES BY CHEMICAL MEANS

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INTRODUCTION

IN MY opinion, typical early caries of the enamel is characterized by (1) change in color, to either white or brown; (2) softening of the enamel, as determined by pressure with a sharp metal probe; (3) radiographic rarefaction of the affected tissue; and (4) in most instances preservation of surface contour and gloss while penetration of the process proceeds toward and into the dentin.

The purpose of this article is to show that similar macroscopic changes are produced in vitro by acid solutions when they contain both calcium and phosphate ions in concentrations which are considerable, but not sufficient to suppress entirely the solution of the enamel. The findings in this report appear to be in general agreement with the observations of Hardwick.[1]

McIntosh,[2] Dietz,[3] Hurst, Frisbie, and Nuckolls,[4] and Besic[5] have demonstrated by in vitro experimentation carieslike changes of the enamel in solutions supporting bacterial growth. Enright, Friesell, and Trescher[6] have shown by chemical methods, at least in some instances, a similarity between their artificially produced enamel changes and true dental caries.

METHOD

Tooth crowns or sections of crowns from freshly extracted teeth were cleaned of debris with a stiff-bristled hand brush. In a few cases any existing stain on the enamel surface was removed with a rotating abrasive-containing rubber disk under water. The tooth crowns or sections were coated with dental stick wax which completely covered them except for a small area of about 4 sq. mm. The use of a small window prevented extensive changes in pH, as well as great changes in the calcium salt concentration as the experiment progressed. In addition, the affected enamel could easily be compared macroscopically with unaffected enamel. During experimentation the specimens were placed in containers in such a manner that the exposed windows were facing upward to permit unaltered exposure to the solution.

PREPARATION OF SOLUTIONS

A solution of hydrochloric acid of maximal calcium and phosphate ion concentration of a pH 3.5 was prepared by adding 10 Gm. of anhydrous $Ca_3(PO_4)_2$ to 500 ml. of HCl of approximately 0.2 N solution. A higher pH was obtained by adding slightly less acid originally. Acetic and lactic acid solutions were prepared in a similar manner. The acetic acid solutions, before additions of

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$Ca_3(PO_4)_2$, were approximately 0.3 N, and the lactic acid solutions were approximately 0.2 N. The final pH of each solution was approximately 4.0. The pH determinations were made by color indicators and, where noted in Table I, potentiometrically with the glass electrode.

A solution of one-half maximal calcium and phosphate ion concentration was prepared by adding one part of distilled water to one part of an already prepared solution of maximal calcium and phosphate ion concentration which has been filtered clear.*

Since this study is concerned essentially with the type of enamel change resulting from exposure to various solutions, rather than a qualitative and quantitative determination of the dissolved enamel salts, only approximate measurement of the penetrating action in each experiment was determined directly with a millimeter (Boley) gauge.

At the conclusion of each experiment observations of any action were made by visual examination with a magnifying lens. An explanation of the symbols used in classifying the type of action on enamel surfaces is given in Table I.

In some instances dental radiographs were taken to designate penetration, and in other cases portions of the affected enamel were removed and penetration was measured directly.

RESULTS

Table I shows that changes of the enamel closely resembling clinical caries can be produced in lactic, acetic, hydrochloric, and carbonic acid solutions of one-half maximal calcium and phosphate ion concentration. Acid solutions containing very low concentrations of calcium and phosphate ions do not produce typical carieslike changes. Such solutions produce changes characterized by a loss of surface contour and gloss without the characteristic partial and penetrating loss of material noted in true early enamel caries. Acid solutions of maximal calcium and phosphate ion concentrations at pH 3.58 or above are incapable of affecting intact enamel in vitro in periods up to five months. Observations for longer exposure times were not made.

Fig. 1, *A* shows typical carieslike enamel changes on the labial surface of a lower canine tooth. The section was coated with dental sticky wax except for a small "window" on the labial surface, and was placed in an acid solution (pH 4.0) of one-half maximal calcium and phosphate ion concentration. The exposure time was twenty days. The result is a change not affecting the original surface contour and gloss. A portion of the affected area was chipped away to show the penetration of the process. Fig. 1, *B* is a roentgenographic view of the lesion showing rarefaction of the altered enamel.

Fig. 2 shows the typical result of exposing an enamel surface to an acid solution with no added calcium phosphate (tribasic). This tooth, a lower left second premolar of unknown origin, was coated with wax except for the window area, and was placed in a lactic acid solution pH 4.0 for ten days. The result is a loss of surface contour. The white powdery residue on the right

*It is difficult to obtain a clear solution without repeated filtration.

TABLE I

TYPE OF CHANGE PRODUCED ON GROSS ENAMEL SPECIMENS EXPOSED TO VARIOUS SOLUTIONS

EXPERIMENT	SOLUTION (VOL. = 400 ML.)	TOOTH AND SURFACE	EXPOSURE TIME	TYPE OF CHANGE	PENETRATION
1	H_2O (tap water from Elmhurst, Ill. Total hardness: 350 ppm, essentially $CaCO_3$). pH 7.5.	Tooth A ($\overline{\lvert 2}$), ♀, age 52, low caries incidence; mesial half of labial surface; also buccal surfaces of $\overline{7\rvert}$; $\overline{\lvert 6}$ (unknown origin).	6 months	–	0
2	H_2O (distilled, in this and succeeding experiments). H_2O changed weekly. pH 6.0—no measurable pH change in one week.	Tooth A, distal half of labial surface; also labial of $\overline{\lvert 3}$, $\overline{\lvert 1}$ (unknown origin).	6 months	+ (Action not uniform, $\overline{\lvert 3}$ more affected than $\overline{\lvert 1}$ and section from tooth A.)	immeasurable
3	H_2O + HCl (solution changed weekly). pH 4.8 (original). pH 5.2 (final-weekly). Potentiometric determinations.	Tooth B ($\overline{6\rvert}$), ♂, age 62, low caries incidence, mesial half of buccal surface; also teeth $\overline{\lvert 4}$, and $\overline{5\rvert}$ buccal surface (unknown origin).	1 month	+ (All three teeth affected approximately the same.)	immeasurable
4	H_2O + HCl + Versene powder (2 Gm.); pH 6.8 (original); pH 7.0 (final). Note: similar results with perhaps greater action at higher pH (8 to 12).	Tooth B, distal half of buccal surface; also tooth $\overline{2\rvert}$, caries-free, labial surface, unknown origin.	2 days	+ (Both teeth affected approximately the same; absence of white powdery residue when specimens dry.)	0.1 mm.
5	H_2O + sodium citrate (.01M). pH 7.0 (original and final).	Tooth C, $\overline{1\rvert}$, ♀, age 58, moderate caries incidence, mesial half of labial surface.	10 weeks	+	0.1 mm.
6	H_2O + citric acid. pH 4.0 (original); pH 5.0 (final).	Tooth C, distal half of labial surface.	10 days	+	0.2 mm.
7	H_2O + lactic acid. pH 4.0 (original); pH 4.8 (final).	Tooth D, $\overline{\lvert 5}$, unknown origin, mesial half of buccal surface coated with DC4 silicone, excess silicone wiped off.	10 days	+	0.1 mm.
		Tooth D, distal half of buccal surface not coated with silicone.	10 days	+ (Action approximately same as coated specimen.)	0.1 mm.
8	Phosphate buffer (1.M). pH 5.0.	$\overline{6\rvert}$, buccal surface; unknown origin.	1 month	⊕ (Very faint action.)	immeasurable
9	H_2O + sodium phosphate monobasic (saturated solution). pH 3.3.	$\overline{7\rvert}$, buccal surface, unknown origin.	10 days	+	0.1 mm.
10	H_2O + $CaCl_2$ solution saturated with excess $CaCl_2$. pH 6.0 (original and final).	Tooth E, $5\rvert$, unknown origin, mesial half of buccal surface.	3 months	–	0

TABLE I—CONT'D

EXPERIMENT	SOLUTION (VOL. = 400 ML.)	TOOTH AND SURFACE	EXPOSURE TIME	TYPE OF CHANGE	PENETRATION
11	H_2O + HCl + $CaCl_2$ (saturated with excess $CaCl_2$). pH 3.5 (original); pH 3.8 (final).	Tooth E, distal half of buccal surface.	8 days	+	0.3 mm.
12	H_2O + acetic acid + $Ca_3(PO_4)_2$; saturated with excess $Ca_3(PO_4)_2$. pH 4.0 (original and final). Potentiometric determination.	Tooth F, $\underline{4}$|, ♂ age 56, moderate caries incidence; distal half of buccal surface.	1 month	−	0
13	H_2O + HCl + $Ca_3(PO_4)_2$; saturated with excess $Ca_3(PO_4)_2$. pH 4.0 (original and final). A similar solution at pH 3.58 produced similar result on enamel. pH determination potentiometrically.	Tooth F, mesial half of buccal surface; half of window enamel slightly ground and remaining enamel untouched.	5 months	− (Neither ground nor untouched enamel surfaces affected.)	0
14	H_2O + HCl + $Ca_3(PO_4)_2$ (one-half of maximal calcium and phosphate ion concentration). pH 4.0 (original); pH 4.5 (final).	Tooth F, lingual surface.	1 month	⊕	0.2 mm.
15	H_2O + lactic acid + $Ca_3(PO_4)_2$; saturated with excess $Ca_3(PO_4)_2$. pH 4.0 (original and final). (Solution changed weekly to limit growth of fungi.)	Tooth G, |$\underline{4}$, ♀. Age 28, moderate caries incidence; mesial half of buccal surface ground, distal half untouched.	1 month	− (Neither ground nor untouched enamel affected.)	0
16	H_2O + lactic acid. pH 4.5 (original); pH 5.1 (final).	Tooth H, $\overline{7}$|, ♀. Age 65, moderate caries incidence; mesial half of buccal surface.	2 days	+ (Compare with Experiments 17 and 18.)	0.2 mm.
17	H_2O + lactic acid + $Ca_3(PO_4)_2$. Saturated with excess $Ca_3(PO_4)_2$. pH 4.5 (original and final). (Solution changed weekly.)	Tooth H, distal half of buccal surface.	20 days	−	0
18	H_2O + lactic acid + $Ca_3(PO_4)_2$ (one-half of maximal calcium and phosphate ion concentration). pH 4.5 (original); pH 4.9 (final). Potentiometric determination (solution changed weekly).	Tooth H, lingual surface.	20 days	⊕	0.2 mm.
19	H_2O + acetic acid. pH 4.5 (original); pH 5.0 (final).	Tooth I, |$\overline{7}$, same patient as in Experiment 16; mesial half of buccal surface.	2 days	+ (Compare with Experiments 20 and 21.)	0.2 mm.

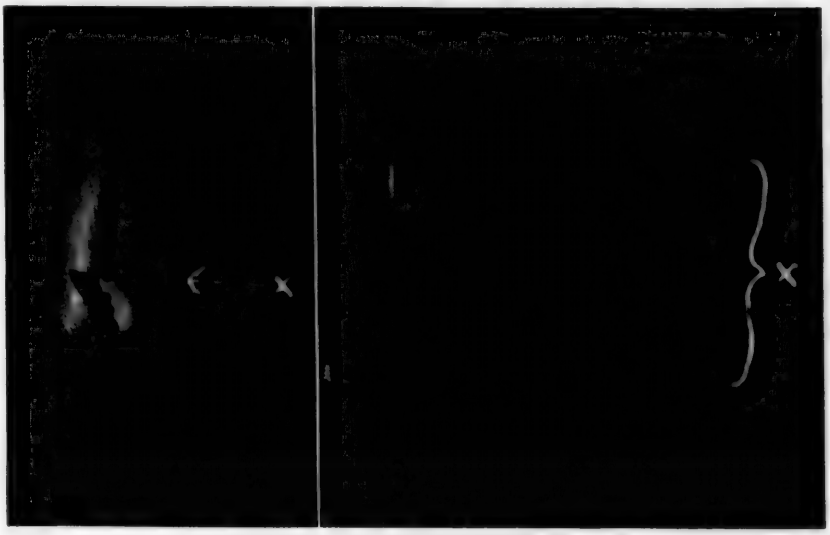
TABLE I—CONT'D

EXPERIMENT	SOLUTION (VOL. = 400 ML.)	TOOTH AND SURFACE	EXPOSURE TIME	TYPE OF CHANGE	PENETRATION
20	H_2O + acetic acid + $Ca_3(PO_4)_2$. Saturated with excess $Ca_3(PO_4)_2$. pH 4.5 (original and final).	Tooth I, distal half of buccal surface.	20 days	–	0
21	H_2O + acetic acid + $Ca_3(PO_4)_2$; one-half maximal calcium and phosphate ion concentration. pH 4.5 (original); pH 5.0 (final).	Tooth I, lingual surface.	20 days	⊕	0.2 mm.
22	H_2O (distilled) + CO_2 (pressure greater than 1 atmosphere). Container consisted of 12-ounce metal beer can with removable cap. pH during experiment undetermined. pH 4.2 (final) determined approximately with color indicators.	Tooth J, $\overline{\lfloor 6}$, ♂, age 57, low caries incidence, mesial half of buccal surface.	1 month	+	0.2 mm.
23	H_2O + CO_2 + $Ca_3(PO_4)_2$ saturated with excess $Ca_3(PO_4)_2$. Other conditions of experiment same as No. 22. pH 6.2 (final).	Tooth J, distal half of buccal surface.	1 month	–	0
24	H_2O + CO_2 + $Ca_3(PO_4)_2$ (one-half of maximal calcium and phosphate ion concentration). Conditions were as above. pH 5.0 (final).	Tooth J, mesial half of lingual surface.	1 month	⊕	0.1 mm.
25	H_2O + CO_2 + $CaCO_3$ (saturated with excess $CaCO_3$); other conditions as above. pH 6.0 (final).	Tooth K, $\overline{7\rfloor}$, ♂, age 58, low caries incidence; mesial half of buccal surface.	1 month	–	0
26	H_2O + CO_2 + $CaCO_3$ (one-half of maximal calcium and carbonate ion concentration); other conditions as above. pH 5.0 (final).	Tooth K, distal half of buccal surface.	1 month	⊕ (Action approximately the same as in Experiment 24.)	0.1 mm.
27	10 c.c. saliva + 1 Gm. sucrose, incubated 24 hours. pH 3.5 (original); pH 3.8 (final).	Tooth L, $\underline{\lfloor 7}$, ♀, age 56, moderate caries incidence; mesial half of buccal surface.	1 month	⊕ (Compare with Experiments 28 and 29.)	0.8 mm.
28	10 c.c. saliva + 1 Gm. sucrose + excess $Ca_3(PO_4)_2$, incubated 48 hours. pH 4.0 (original and final).	Tooth L, distal half of buccal surface.	1 month	–	0
29	10 c.c. saliva + 1 Gm. sucrose, incubated 24 hours; boiled to sterilize, unfiltered. pH 3.5 (original), pH 3.9 (final).	Tooth L, lingual surface. (Section sterilized with 95 per cent ethyl alcohol.)	1 month	⊕	0.8 mm.

Symbols (Column 5):
+ = loss of surface continuity (surface of exposed window depressed); loss of surface gloss; fine white powdery residue remaining on dried specimen.
⊕ = original surface gloss and contour unaltered; affected area opaque and softened. This type resembles changes seen in caries of enamel.
– = no apparent macroscopic change.
Penetration = approximate measurement in millimeters from original surface level to hard unaffected enamel.
All experiments were conducted at room temperature, and all teeth were from people of the white race.

side of the window was wiped off to expose hard unaffected enamel immediately below. This type of action is not typical of natural caries of the enamel.

Fig. 3 shows that in an acid solution of one-half maximal calcium and phosphate ion concentration different teeth, as well as different areas of the same tooth, show variations in susceptibility to dissolution. This finding gives credence to the belief that the character of enamel structure may be an important factor in dental caries.



A. *B.*

Fig. 1.—Window area (*x*) in *A* was exposed to H_2O + HCl + $Ca_3(PO_4)_2$, pH 4.0 one-half of maximal calcium and phosphate ion concentration. Exposure time, twenty days. Note carieslike changes of enamel in window area; portion of affected area chipped off to show penetration. This type of enamel change is denoted by the symbol ⊕ in Table I.

B is a roentgenograph of the section in *A* showing radiolucency in window area (*x*).

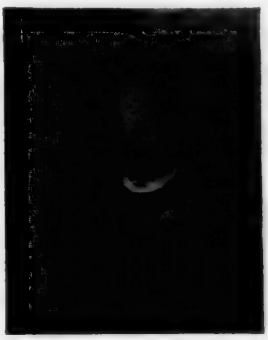
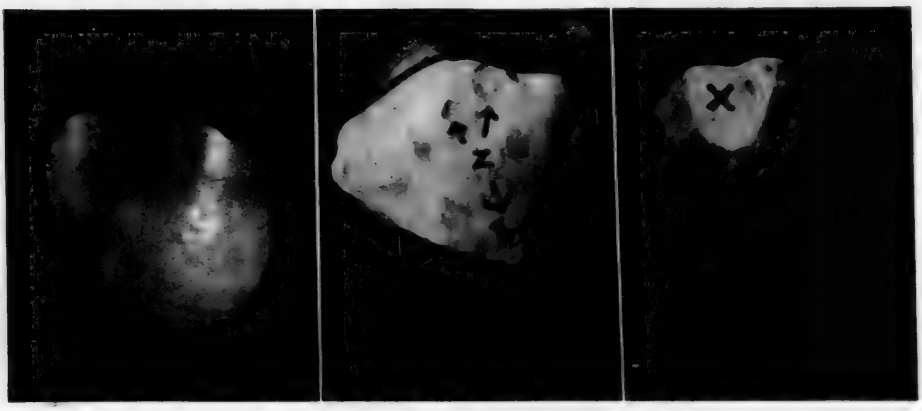


Fig. 2.—Window area (*x*) was exposed to H_2O + lactic acid, pH 4.0, for ten days. Note affected area is totally unlike caries of enamel. White powdery residue on right side of window was wiped off to show hard unaffected enamel below. The type of enamel change shown in this photograph is denoted by symbol + in Table I.

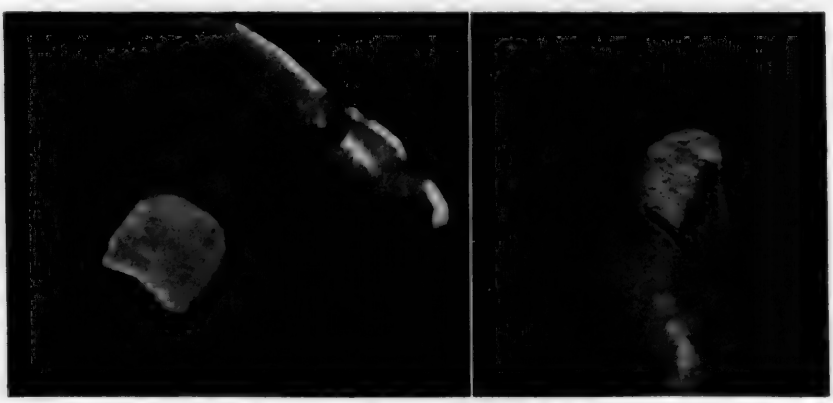
Fig. 4 shows the result of exposing the enamel surfaces of two teeth to a carbonic acid solution with no added calcium phosphate (or carbonate). These photographs show a loss of surface contour.



A. *B.* *C.*

Fig. 3.—Crowns of three caries-free teeth were covered with wax, except outlined window areas. Each tooth was extracted from a different female patient past 30 years of age with low caries activity and moderate past caries experience. All three crowns were exposed to a solution of H_2O + HCl + $Ca_3(PO_4)_2$, pH 3.8 (one-half of maximal calcium and phosphate ion concentration) in a common container for two days.

Crown *A*, a lower right second premolar, shows faint irregular carieslike changes. The pronounced white area is due to reflected light showing retention of surface gloss. Crown *B*, a lower right second premolar, shows greater and more diffused carieslike changes and only a few isolated (dark) unaffected areas. *Z* areas were chipped to show some penetration even at two days' exposure. Crown *C*, a lower left first premolar, shows generally the same appearance as Crown *A* except at the area of natural attrition (*X*) which shows a diffuse carieslike change.



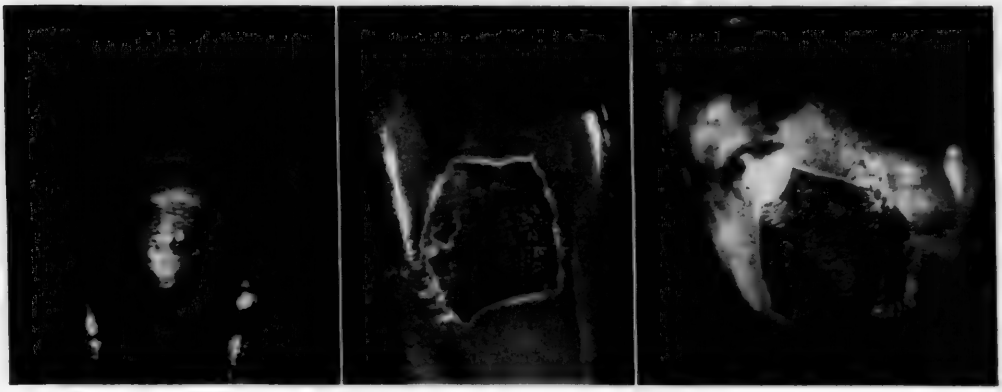
A. *B.*

Fig. 4.—Window areas of two teeth exposed for three weeks in distilled H_2O + CO_2 (pressure greater than 1 atmosphere). Notice loss of surface contour and absence of other characteristics of clinical caries of enamel. Similar enamel changes (but at a slightly slower rate) were observed on other specimens exposed to distilled water saturated with CO_2 at 1 atmosphere of pressure.

Fig. 5 shows the result of exposing the enamel surfaces of three teeth to a carbonic acid solution of one-half maximal calcium and phosphate ion concen-

tration. The affected areas resemble natural caries of enamel. Surface contour is retained in all three teeth and penetration is shown by the mechanically removed portion of affected tissue in Tooth *C*.

Fig. 6 shows two photographs of a sectioned lower incisor. The tooth had been coated with wax, except for a window on the labial surface, and subjected to



A. *B.* *C.*

Fig. 5.—Three teeth from three different patients were coated with wax, except in window areas, and immersed in $H_2O + Ca_3(PO_4)_2 + CO_2$ (one-half of maximal calcium and phosphate ion concentration before addition of CO_2) at a pressure greater than 1 atmosphere for three months. Solution was renewed monthly. Note preservation of surface features on all three teeth and penetration in chipped portion (*x*) of window on Tooth *C*.

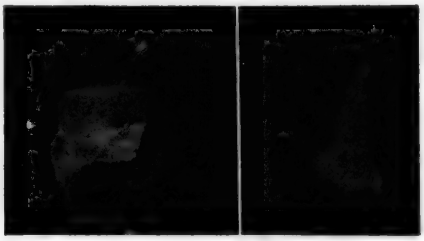
A. *B.*

Fig. 6.—Photographs after sectioning of a lower incisor which previously was treated in $H_2O + Ca_3(PO_4)_2 + CO_2$ (one-half of maximal calcium and phosphate ion concentration before addition of CO_2) at a pressure greater than 1 atmosphere for eight months, the solution being renewed monthly. *A* is a section cut through center of window area. Note penetration through enamel into dentin. *B* is a roentgenograph of section *A* showing rarefaction in enamel and dentin. Note lack of spreading process at dentinoenamel junction. The affected enamel in the window area showed (before sectioning) preservation of surface contour and gloss until the seventh month of exposure. Unfortunately, no photograph was made at that time.

a carbonic acid solution of one-half maximal calcium and phosphate ion concentration for eight months. A section through the affected area shows penetration of the process into the dentin. A roentgenographic view of the section shows

rarefaction of the enamel and dentinenamel junction and no indication of any cone-shaped rarefaction toward the pulp chamber, as is characteristic of the true lesion. The experimental conditions, which obviously limited any significant bacterial activity and omitted all in vivo influences such as would occur from a vital pulp, probably account for such atypical activity in the dentin.

Whole saliva acidified by bacterial activity and which contained less than maximal calcium and phosphate ion concentration produced characteristic carieslike enamel changes (Experiments 27 and 28, Table I). Saliva, acidified by bacterial activity and containing a maximal calcium and phosphate ion concentration at pH 4.0, had no effect on intact enamel in one month; Fig. 7 shows the result of Experiment 29.



A. *B.*

Fig. 7.—Result obtained in Experiment 29, Table I. The section in *A* (with wax window technic) was exposed for one month to a solution consisting of 10 c.c. saliva + 1 Gm. sucrose, previously incubated for twenty-four hours to acidify, boiled to sterilize and unfiltered. The waxed section with window was wiped with 95 per cent ethyl alcohol several times to approach sterility before exposure to the solution. The pH of the solution was 3.5 at beginning of experiment and 3.9 at conclusion. *A* shows the window with carieslike changes. *B* is a roentgenographic view of the section in *A*, showing rarefaction of the enamel in the window area.

DISCUSSION

Some of the observations recorded in this paper are in apparent disagreement with certain observations of Enright, Friesell, and Trescher.[6] Of the enamel changes produced by them certain specimens apparently showed the closest similarity to natural caries, these specimens being produced in acid solutions (pH below 5.0) which they characterize as "previously saturated with tricalcium phosphate." It is to be noted that because of the manner of preparation these solutions did not have maximal calcium and phosphate ion concentration when the enamel was exposed to them because (p. 798 of their description) they acidified their solutions *after* saturation with $Ca_3(PO_4)_2$. Thus, their report appears to indicate that solutions with maximal calcium and phosphate ion concentrations below pH 5.0 merely reduce the solubility of enamel, while the data in this report indicate a total inhibition of any enamel change in such acid solutions with pH as low as 3.5.

The description of caries by Enright, Friesell, and Trescher also is not in agreement with the characterizations given in this article. They stated, "When caries attacks the enamel, the entire carious area is dissolved, because there is so little organic matter in it that it cannot maintain its form, and a cavity results." This statement is in disagreement with my views and those of

other clinicians who believe the primary change of the enamel in most cases of natural caries is a softening of the tissue which is lost secondarily by mechanical disintegration. Clinically, early carious lesions usually show normal surface contour of the affected enamel and in many instances where mechanical manipulation (by tooth brushing, mastication, dental operations, etc.) has not occurred, the original surface gloss is nearly always retained. In some few instances the enamel surface is seen to be dull.

SUMMARY

1. Typical macroscopic characteristics of true caries of human enamel appear to have been duplicated in vitro by the action of a number of acid solutions containing calcium and phosphate ions in less than maximal concentration.

2. The same acid solutions with low concentrations of calcium and phosphate ions produced on enamel surfaces changes which do not resemble natural caries.

3. The same acid solutions with maximal calcium and phosphate ion concentration (in some cases at pH 3.58) appear to produce no effect on enamel in vitro.

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THE AMOUNTS OF FOLIC ACID AND VITAMIN B_6 IN SALIVA

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THE vitamin content of saliva is of interest from a number of standpoints. The present work is concerned with the amounts of two of the vitamins which influence growth of some oral lactobacilli. It was prompted by information now available concerning which of the vitamins are needed for growth of these microorganisms and the limitations imposed upon their growth and acid production by suboptimum levels of a required vitamin. Does saliva contain enough of each of the vitamins required by oral lactobacilli to support sufficient growth and acid production for attainment of a pH level in the 4.0 to 4.5 range?

General experience with the several lactobacilli used for microbiologic assays and also information from a detailed study of some oral lactobacilli[1] have shown that very small amounts of required vitamins often are sufficient to support rather extensive cell metabolism, growth, and acid production. Although these studies apply more particularly to pure cultures in laboratory media where ample supplies of other nutritive substances are available, the information is nevertheless of some interest from the standpoint of activity of these organisms in the mouth where numerous other factors may influence multiplication of microorganisms.

Few attempts have been made to study the vitamin content of saliva, particularly with respect to those vitamins needed by lactobacilli. Kniesner, Mann, and Spies[2] found that the pantothenic acid content of saliva of fifty-one persons averaged 0.088 μg per milliliter. Glavind, Granados, Hansen, Schilling, Kruse, and Dam[3] studied the saliva of eight young adults; the amounts of vitamins of the B group which they list, in terms of micrograms per milliliter of saliva, are: thiamine as the hydrochloride 0.007, riboflavin 0.05, nicotinic acid 0.03, pantothenate as the Ca salt 0.08, biotin 0.0008, folic acid 0.0001, and pyridoxine as the hydrochloride 0.6. In a later study, Granados, Glavind, Noer, and Dam[4] reported the presence of vitamin B_{12} in two samples of human saliva; one contained 0.005 μg per milliliter and the other 0.0015 μg.

Since the amounts of folic acid and of pyridoxine reported for saliva seemed somewhat out of line with the relationship in amounts of the vitamins as reported for various tissues and blood, it was decided to investigate further the salivary content of these two vitamins. The results form the subject of this report.

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FOLIC ACID (PTEROYLGLUTAMIC ACID)

Assay Procedure.

Microorganism: The organism used was a homofermentative oral lactobacillus, Number 1009, from the laboratory stock collection. From previous tests[5] it was known to require folic acid for growth. For the purpose of this study the use of an oral strain seemed preferable to either *Lactobacillus casei* ATCC 7469 or *Streptococcus faecalis* R ATCC 8043, the two organisms usually recommended for folic acid assays.[6] Since different lactic acid bacteria respond differently to substances with folic acid activity (i.e., pteroyltriglutamic acid, vitamin Bc conjugate or the pteroylheptaglutamate, the SLR factor, thymine, and others), an oral lactobacillus seemed preferable for determining the amounts of folic acid and substances with folic activity in saliva which might influence the growth of oral lactobacilli. The assay organism was kept in stock on tomato juice-yeast extract (TJY) broth with transfers every three to four weeks.

Basal medium: The composition of the basal medium used for the tests is given in Table I, which shows the concentrations of the compounds of the final medium as used in the assays. When preparing the medium it was customary to make it double strength, dissolving the ingredients in one-half the indicated amount of distilled water. When used in the assays, 5 ml. of double strength basal medium in each tube were made up to a total of 10 ml. with the sample and distilled water, so that the final concentration of ingredients was that shown in the table. All vitamins except riboflavin were autoclaved in the medium. Riboflavin was sterilized separately by filtration and added after autoclave sterilization.

TABLE I

COMPOSITION OF BASAL MEDIUM

COMPONENT	QUANTITY PER 1,000 ML.	COMPONENT	QUANTITY PER 1,000 ML.
Acid-hydrolyzed casein	10 gm.	K_2HPO_4	1.0 gm.
Glucose	20 gm.	NaCl	10.0 gm.
L-cystine	100 mg.	$MgSO_4 \cdot 7H_2O$	200.0 mg.
L-tryptophane	100 mg.	$FeSO_4 \cdot 7H_2O$	10.0 mg.
DL-threonine	100 mg.	$MnCl_2 \cdot 4H_2O$	7.5 mg.
DL-serine	100 mg.	Nicotinic acid	1.0 mg.
L-hydroxyproline	100 mg.	Ca pantothenate	1.0 mg.
Guanine	10 mg.	Pyridoxal·HCl	1.0 mg.
Xanthine	10 mg.	Thiamine·HCl	500.0 μg
Uracil	10 mg.	Biotin	10.0 μg
Na acetate·$3H_2O$	10 gm.	Riboflavin	200.0 μg
KH_2PO_4	1 gm.		

Adjusted to pH 6.8.

Inoculum: A twenty-four-hour culture in TJY broth was twice centrifuged and resuspended in an amount of buffered saline equivalent to the original volume of medium. From this 0.1 ml. was transferred to 10 ml. sterile saline and then 0.1 ml. of this dilution was used to inoculate each tube of the test. This amount of washed inoculum is equal to approximately 0.001 ml. of original culture. The alternate method of growing the organism in the basal medium

plus a small amount of folic acid was also used in some of the earlier tests, but seemed to offer no advantage. Since the use of the TJY medium was somewhat simpler and at the same time gave satisfactory blanks in control tubes (without folic acid), it was employed in most of the tests.

Saliva samples: Most of the saliva specimens were collected by paraffin stimulation between 9:00 and 10:00 A.M., usually about one to two hours after breakfast. About 10 ml. of saliva was collected. The specimens were then used immediately in setting up the tests; usually not more than thirty to forty-five minutes elapsed.

Procedure: After thorough mixing, graded amounts of saliva were added to duplicate clean Pyrex test tubes, 18 by 150 mm. The volume was made up to 5 ml. with distilled water, and 5 ml. of double strength basal medium was added per tube. The tubes were plugged with cotton wrapped in gauze and autoclaved at 15-pound pressure for ten minutes. After cooling, riboflavin was added aseptically, the tubes were inoculated, and incubated at 37 degrees for forty-eight hours, or in some cases for seventy-two hours. Growth response was determined by the amount of acid produced, using 0.1 N NaOH for titration. This method was preferable to the use of turbidity, since some of the larger amounts of saliva contributed turbidity to the medium. With each test, folic acid dilutions were also set up for the standard curve. Amounts of folic acid in micrograms per tube were: 0, 0.0001, 0.0005, 0.001, 0.002, 0.005, 0.007, 0.01, 0.05, and 0.1. These tubes were treated in exactly the same manner as the saliva-containing tubes with respect to volume of basal medium, sterilization, and other procedures.

A standard curve was plotted on semilog paper, using titratable acid against micrograms of folic acid per standard tube. Results from different amounts of each saliva specimen were calculated in terms of micrograms of folic acid per milliliter of saliva. Tubes which gave readings near the lower or upper part of the curve were not included in the results. A test was regarded as satisfactory if it gave a 15 per cent check on at least two dilutions of saliva, though in many instances this was obtained with three dilutions.

One point of departure from suggested methods[6] of folic acid assay should be noted. Digestion of the saliva samples with the use of enzyme from chicken pancreas or hog kidney was purposely omitted, although this procedure is recommended for microbiologic assay of foodstuffs and similar products for the purpose of liberating bound folic acid. Lactobacilli do not respond or they respond poorly to some of the bound forms of the vitamin, such as vitamin Bc conjugate. Since our purpose was to measure the amount of folic acid, or substances with folic acid-like activity, which would stimulate the metabolic activity of oral lactobacilli, the enzyme digestion of the samples was omitted. That is, had enzyme digestion been used with resulting liberation of folic acid from any of its conjugates that might occur in saliva, the values obtained might well have included some substances which are ordinarily not available to oral lactobacilli.

Results.—In Table II are summarized the results of assays of fifty-one saliva samples from twenty persons, nine men and eleven women. The subjects were

chosen at random without respect to their caries experience; the majority of them were in the age group of 25 to 35 years, with about one-fourth between 45 and 55 years. The samples were collected at varying times over a three-month period. Each figure is the average of values obtained from at least two dilutions of a saliva sample. The calculated values for different dilutions of each sample checked within 15 per cent, and the average variation was less than 10 per cent.

TABLE II

VALUES FOR FOLIC ACID AND SUBSTANCES WITH FOLIC ACID ACTIVITY IN SALIVA

PERSON	μG/ML.	PERSON	μG/ML.	PERSON	μG/ML.
1	.009	8	.046	14	.014
	.013		.005		.007
2	.026	9	.008	15	.024
	.039		.008		.013
	.009				.008
	.012	10	.006		
	.011		.027	16	.023
			.003		.018
3	.012				
	.018	11	.013	17	.049
			.017		.019
4	.019		.026		
	.021		.016	18	.061
					.020
5	.017	12	.044		
	.028		.075	19	.006
			.050		.012
6	.059		.019		
	.049		.054	20	.013
			.045		.022
7	.010				
	.024	13	.018		
			.028		

Average: .024 μg per milliliter. Extreme range: .003 to .075 μg per milliliter, with 70 per cent of the values between .008 and .039 μg per milliliter.

While in most cases there was little difficulty in obtaining values which checked within reasonable limits, a few instances occurred in which tests were obviously unsatisfactory. For a few saliva samples it proved to be impossible to obtain figures which could be deemed reasonably accurate. In the entire series of tests there were eight samples of saliva which showed only slight growth of the test organism in all tubes, irrespective of the varying amounts of saliva which had been added. Whether the saliva in these cases may have contained some inhibitive or antibacterial substance after the usual autoclaving is a matter of conjecture; the reason for such results was not apparent. They are not included with the results in Table II, since no reliable figures could be obtained in these several instances.

The average figure for all of the fifty-one specimens from twenty persons was 0.024 μg folic acid, or substances with folic acid activity, per milliliter of saliva. Most of the samples gave values between 0.008 and 0.039 μg, although the extremes were 0.003 and 0.075 μg per milliliter. One feature of the results was the variability in the figures, not only for different persons, but for the same individual upon repeated tests at different times. This could be attributed

to uncertainties of the microbiologic method of assay, to actual fluctuations in the level of the vitamin and related active substances in saliva, or to varying combinations of the foregoing factors. The checks on the method of assay will be presented later. Some fluctuation, and perhaps considerable fluctuation, of the amount of vitamin in saliva might reasonably be expected, depending upon the condition of the mouth, diet, the possible presence of other microorganisms capable of synthesizing the vitamin, vitamin destruction, perhaps previous activity or inactivity of the salivary glands, and other factors. It may be noted that, in studies of the amounts of pantothenic acid in saliva, Hill and Kniesner[7] reported values ranging from 0.008 to 0.17 μg per milliliter in fifteen saliva specimens, and Kniesner, Mann, and Spies[2] found values ranging from 0.012 to 0.19 μg per milliliter of saliva in the examination of fifty-one persons. Thus, considerable variation in the amount was also encountered in these instances.

A comparison of our average figure for folic acid active substances with the values found for blood and milk is of interest. Denko, Grundy, and Porter,[8] in examining seven men, obtained an average of 0.025 μg folic acid per milliliter of whole blood and 0.017 μg per milliliter of plasma. Assays of folic acid applied to milk have presented some unusual difficulties, as mentioned, for example, by Hobson,[9] and the published figures vary considerably. Williams, Eakin, Beerstecher, and Shive[10] give the figure for human milk as 0.11 μg per milliliter, with a range of 0.08 to 0.13 μg per milliliter. Collins, Harper, Schreiber, and Elvehjem[11] found much smaller amounts in human milk (five persons); the mean was 0.0007 μg per milliliter with a range of 0.0001 to 0.0018 μg per milliliter. For cow's milk the mean was 0.0013 μg per milliliter with a range of 0.0002 to 0.004 μg per milliliter.

TABLE III

UNIFORMITY OF ASSAY VALUES OBTAINED WITH DIFFERENT AMOUNTS OF THE SAME SPECIMEN

AMOUNT OF SALIVA (MILLILITERS)	NUMERICAL DESIGNATION OF THE PERSON (MICROGRAMS OF FOLIC ACID ACTIVITY FOUND PER MILLILITER OF SALIVA)							
	3	4	5	6	10	12	16	18
.050			.030	.048		.048		
.075			.029	.049		.045		
.100	.011	.025	.028	.051	.013	.044	.020	.015
.150	.009	.022	.029	.042	.015	.044	.024	.021
.200	.013	.024	.026	.055	.016	.038	.022	.023
.250	.012	.023		.050			.024	.021
.300	.014	.023			.019		.024	

The reliability of the assay procedure was tested in two ways: first, the constancy of results when different amounts of the same sample were assayed and, second, the addition of known amounts of folic acid to saliva samples with estimates of the per cent recovery and the total amount. Table III gives figures for folic acid activity calculated from several different amounts of each of eight saliva specimens. Table IV summarizes the results of tests in which known amounts of folic acid were added to saliva samples. The figures for recovery of added folic acid ranged from 80 to 140 per cent with one exception; in this one case the amount added was small in relation to the total present. Expressed in terms of the proportion of total folic acid activity found by assay to the

theoretical total, the values ranged from 91 to 109 per cent. The results given in both Tables III and IV indicate that the assay method gives a reasonably reliable idea of the amount of substances with folic acid activity that stimulates growth of lactobacilli.

TABLE IV

RESULTS OF THE ADDITION OF FOLIC ACID TO SALIVA SAMPLES WITH SUBSEQUENT ASSAYS

SALIVA SAMPLE	AMOUNT OF SALIVA (ML.)	FOLIC ACID ACTIVITY FOUND BY ASSAY (μG)	ADDED FOLIC ACID PER TUBE (μG)	THEORETICAL TOTAL (μG)	TOTAL FOUND BY ASSAY (μG)	RECOVERY OF ADDED FOLIC ACID (PER CENT)	TOTAL FOUND BY ASSAY TO THEORETICAL TOTAL (PER CENT)
1	.10	.001	.001	.002	.002	100	100
2	.15	.0030	.002	.0050	.0054	120	108
	.20	.0047	.002	.0067	.0070	115	104
	.25	.0072	.001	.0082	.0076	40	93
3	.30	.0082	.001	.0092	.0096	140	104
4	.10	.0013	.002	.0033	.0030	85	91
	.15	.0023	.002	.0043	.0047	120	109
	.20	.0033	.002	.0053	.0056	115	106
	.30	.0058	.002	.0078	.0080	110	103
5	.05	.0024	.002	.0044	.0044	100	100
	.075	.0037	.002	.0057	.0061	120	107
	.10	.0051	.002	.0071	.0068	85	96
	.15	.0064	.002	.0084	.0080	80	95

VITAMIN B_6

Assay Procedure.—Assays for vitamin B_6 were made using essentially the same microbiologic procedure as for folic acid, with some minor changes.

Microorganism: An oral lactobacillus, Number 46, from our laboratory collection was chosen as the assay organism. This strain requires vitamin B_6 for growth in a casein digest basal medium. The same considerations as those discussed in the section on folic acid led us to use an oral lactobacillus, rather than one of the microorganisms usually recommended for vitamin B_6 assay.[12] In making such a choice, certain points must be kept in mind. It is known from previous work that lactobacilli respond very differently to the different forms of vitamin B_6; pyridoxine shows relatively little or no activity, pyridoxamine is often distinctly less active than pyridoxal which usually possesses the greatest activity of these three forms of vitamin B_6 for lactobacilli.[13, 14] Pyridoxamine phosphate and pyridoxal phosphate are reported to be less active than pyridoxal for *L. casei*,[15] but, on the other hand, an occasional strain of lactobacillus requires a phosphorylated form of vitamin B_6 for growth, or grows more readily in its presence.[14, 16] Relatively few lactobacilli have been thoroughly studied with respect to their comparative response to various vitamin B_6 compounds. The same is true of other groups of microorganisms, although it is known that different organisms behave quite differently.

The foregoing considerations serve to bring out a number of the uncertainties attending the assay of vitamin B_6. Another uncertainty is the lack of knowledge of the form or forms of vitamin B_6 which may occur in saliva and

of their relative proportions. Since, however, our primary aim was the assay of substances showing vitamin B_6 activity which would stimulate growth and acid production of lactobacilli, the use of an oral lactobacillus was decided upon. The strain used for this purpose responds more readily to pyridoxal than to pyridoxamine; pyridoxine shows little activity. Pyridoxal hydrochloride was used as the standard for reference in the assays.

Basal medium: Except for a few changes, the medium was the same as that used for the folic acid assay. Pyridoxal hydrochloride (vitamin B_6) was, of course, omitted, 0.1 μg per milliliter of folic acid and the same amount of para-aminobenzoic acid were added, thiamine was increased from 0.5 μg to 1.0 μg per milliliter. The riboflavin was added to the medium before sterilization, rather than after autoclaving, since it was found that such a procedure did not affect the results.

Inoculum: The procedure was the same as that described for folic acid assay except that 0.002 ml. of washed culture, rather than 0.001 ml., was used to inoculate the tubes. Satisfactory blanks were obtained with the larger inoculum; that is, the amount of vitamin carried over with the cells was not too great.

Saliva samples: All of the saliva specimens were collected by paraffin stimulation, usually about two to three hours after breakfast. About 10 ml. of saliva was collected. The specimens were used immediately in setting up the tests.

Procedure: The placing of graded amounts of saliva samples in test tubes, addition of double strength basal medium, the range of the pyridoxal hydrochloride standards, autoclaving, incubation, titration with standard NaOH solution, and calculation of results from a standard curve were essentially the same as described for folic acid. The saliva samples were not subjected to enzyme digestion or to acid hydrolysis for the reasons discussed earlier.

In the first part of the work some difficulty was experienced in getting reasonably close assay values with different quantities of the same sample, and also in recovery experiments after addition of a known amount of pyridoxal hydrochloride. Certain precautions tended to minimize these difficulties. The sample of pyridoxal hydrochloride used to make up the stock solution was vacuum-desiccated under refrigeration for at least twenty-four to forty-eight hours before being weighed and used. The stock solution was protected from light and was held not more than twenty-four hours before use in making up dilutions for the standards. All tubes in the test, whether containing the standards or saliva, were protected from light as much as possible before and after inoculation.

Results.—Table V summarizes the results of assays of fifty-two saliva samples from seventeen persons, thirteen men and four women. The subjects were chosen at random without respect to their caries experience; the majority of them were in the age group of 25 to 35 years. The samples were collected at varying times over a period of approximately four weeks. Each figure is the

average of values obtained from at least two dilutions of a given sample. The calculated values for the different dilutions of each sample checked fairly well.

TABLE V

VALUES FOR VITAMIN B_6 AND SUBSTANCES WITH B_6 ACTIVITY IN SALIVA

PERSON	μG/ML.	PERSON	μG/ML.	PERSON	μG/ML.
1	.007	4	.003	9	.004
	.003		.001		.003
	.005		.002		
	.006		.002	10	.004
	.006		.002		.006
	.007				
	.009	5	.017	11	.001
	.004		.013		.003
	.006		.015		
			.014	12	.005
2	.011		.010		
	.016			13	.008
	.011	6	.005		
	.008		.002	14	.003
	.008		.003		
	.012		.005	15	.003
3	.004	7	.008	16	.005
	.002		.011		
	.003		.012	17	.003
	.002				
	.004	8	.006		
			.003		
			.005		

Average .006 μg per milliliter.

Extreme range .001 to .017 μg per milliliter, with 75 per cent of the values between .003 and .012 μg per milliliter.

There was little difficulty in obtaining values which checked within reasonable limits, although, as in the previous tests, there were a few instances in which assays were obviously unsatisfactory. These unsatisfactory tests were omitted from the table since no reliable figures could be obtained. In some cases there appeared to be some inhibitive or antibacterial agent present resulting in lesser growths as the amounts of saliva were increased, while in others there seemed to be a stimulatory agent causing increased amounts of growth over that which would normally be expected with the increase in the amount of saliva.

The average figure for the fifty-two samples of saliva was 0.006 μg per milliliter of B_6 activity using pyridoxal hydrochloride as the reference standard. Seventy-five per cent of the samples gave values between 0.003 and 0.012 μg, although the extremes were 0.001 to 0.017 μg per milliliter. It should be noted that there was some variability in the figures between repeated tests at different times on the same individual, as well as between different individuals. Such variability, it would seem, might reasonably be expected.

The reliability of the assay was tested by the same procedures employed for folic acid. Table VI gives figures showing the uniformity of results with several different amounts of each of eight different saliva samples. Table VII summarizes the results of assays in which a known amount of pyridoxal hydrochloride was added to varying amounts of saliva samples. The values obtained

here, as well as the uniformity of the figures for the different saliva concentrations, indicate that the method of assay is reasonably accurate.

The values obtained for both folic acid and vitamin B_6 in saliva may be considered in relation to growth and acid production of oral lactobacilli. In a previous study[1] it was found that folic acid in concentrations of 0.01 to 0.003 μg per milliliter of culture medium supported maximal or almost maximal growth and acid production. From this it would appear that the amounts of folic acid found in saliva in the present study are sufficient for either maximal or near maximal growth and acid production. Interpretation of the results secured with vitamin B_6 is made somewhat more difficult because of the varying activity of different lactobacilli toward different forms of vitamin B_6, and our lack of knowledge of the form or forms of vitamin B_6 which occur in saliva.

TABLE VI

ASSAY VALUES FOR VITAMIN B_6 OBTAINED WITH DIFFERENT AMOUNTS OF THE SAME SPECIMEN

AMOUNT OF SALIVA (ML.)	NUMERICAL DESIGNATION OF THE PERSON (MICROGRAMS OF B_6 ACTIVITY FOUND PER MILLILITER OF SALIVA)							
	1	2	3	6	7	9	10	16
0.1	.007	.008	.0027	.0026	.012	.004		
0.2	.006	.008	.0029	.003	.011	.0039	.004	.005
0.3	.0063	.008	.0027	.003	.011	.004	.004	.0045
0.4	.0067	.008	.0025	.0032		.0042	.0038	.0052
0.5	.0064	.0088	.0032	.0032		.004	.0038	.0048

TABLE VII

RESULTS OF THE ADDITION OF PYRIDOXAL HYDROCHLORIDE TO SALIVA SAMPLES WITH SUBSEQUENT ASSAYS

SALIVA SAMPLE	AMOUNT OF SALIVA (ML.)	B_6 ACTIVITY FOUND BY ASSAY (μG)	ADDED PYRIDOXAL HYDROCHLORIDE PER TUBE (μG)	THEORETICAL TOTAL (μG)	TOTAL FOUND BY ASSAY (μG)	RECOVERY OF ADDED PYRIDOXAL HYDROCHLORIDE (PER CENT)	TOTAL FOUND BY ASSAY TO THEORETICAL TOTAL (PER CENT)
1	.1	.0005	.001	.0015	.0015	100	100
	.2	.001	.001	.002	.002	100	100
	.3	.0017	.001	.0027	.0025	80	93
	.4	.0023	.001	.0033	.0031	80	94
2	.3	.001	.001	.002	.002	100	100
	.4	.00135	.001	.00235	.00232	97	99
	.5	.0018	.001	.0028	.0028	100	100
3	.2	.00098	.001	.00198	.0021	112	106
	.3	.0015	.001	.0025	.0025	100	100
	.4	.0021	.001	.0031	.0029	80	94
	.5	.0024	.001	.0034	.0032	80	94
4	.1	.0017	.001	.0027	.0026	90	96
	.2	.0029	.001	.0039	.0042	130	108
5	.2	.0014	.001	.0024	.0025	110	104
	.3	.0015	.001	.0025	.0027	120	108
	.4	.002	.001	.003	.0031	110	103
6	.1	.00062	.001	.00162	.0016	98	99
	.2	.0012	.001	.0022	.0022	100	100
	.3	.002	.001	.003	.0028	80	93
	.4	.0032	.001	.0042	.0041	90	98

It seems probable, however, that the amount of vitamin B_6 in saliva is sufficient in many cases for definite growth and acid production; in some instances it might well be sufficient for maximal, or almost maximal, growth and acid production of those oral lactobacilli which need this vitamin.

SUMMARY

Microbiologic assays of the saliva content of folic acid and of vitamin B_6, including substances with folic acid-like or vitamin B_6-like activity that stimulate growth of oral lactobacilli gave the following results:

For folic acid, analyses of fifty-one saliva samples from twenty persons, mostly young adults chosen without regard to their caries experience, gave an average figure of 0.024 μg per milliliter. The extreme range was 0.003 to 0.075, with 70 per cent of the values falling between 0.008 and 0.039 μg per milliliter.

For vitamin B_6, using pyridoxal hydrochloride as the standard, analyses of fifty-two saliva samples from seventeen persons in the same category as above, gave the average figure of 0.006 μg per milliliter. The extreme range was 0.001 to 0.017, with 75 per cent of the values falling between 0.003 and 0.012 μg per milliliter.

The relationship of these values to possible growth and acid production of oral lactobacilli has been discussed.

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INVESTIGATIONS ON THE OCCURRENCE AND SIGNIFICANCE OF CITRIC ACID IN THE SALIVA

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CITRIC acid has long been recognized as one of the possible chemical causes of dental erosion.[30, 31] Numerous experiments with animals have shown that the citrate-rich fruit juices have an erosive effect on enamel.[19-23, 30, 31] The most obvious mechanism of this effect is an acid dissolution of the salts of the teeth, since orange, grapefruit, and pineapple juices have a reported pH of 3.8 to 4.0.[20, 23] In addition, the buffering effect of the citrate ions at a low pH produced by this or other acids may play a part. However, it has also been reported that practically neutral citrate solutions have an erosive effect on the teeth of experimental animals.[22] It therefore seems possible that the calcium-combining property of the citrate ions may also have a role in dental erosion by increasing the solubility in the saliva of the calcium salts of the tooth. This latter concept has been supported by the reports of increased solubility of calcium carbonate and calcium phosphate in the presence of citrate.[10, 16, 24]

The purposes of this investigation have been to study certain features of salivary citrate content and to determine the possible effects of intraoral concentrations of citrate on the solubility of calcium phosphate in saliva. Accordingly, this report is divided into two parts: (1) determination of the variations with time of citrate concentrations in saliva after ingestion of a citric acid-containing food and beverage; and (2) determination of the solubility of the enamel apatite in saliva in the presence of comparable concentrations of citrate.

1. THE INTRAORAL CITRATE RETENTION

Methods.—Samples of unstimulated mixed saliva were collected before and at 2, 5, 10, and 20 minutes after the ingestion of an orange or 100 ml. grapefruit juice. The foods were ingested in portions during a 1-minute period. The first saliva sample had a volume exceeding 1 ml. and was taken at least one hour after the last previous meal. The saliva collections made after the food ingestion were obtained in a standard manner with the subjects resting quietly. One and one-half minutes after completing the ingestion, the subject was asked to swallow and then to let the saliva passively collect in the mouth. After 2.5 minutes the saliva was delivered into a beaker. The 2-minute sample was thus the saliva collected over the 1.5 to 2.5-minute period after the completed ingestion, the 5-minute sample at 4.5 to 5.5 minutes, and so on.

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Citrate was determined according to a modification of the pentabromacetone method as applied to the saliva by Zipkin and McClure.[30*]

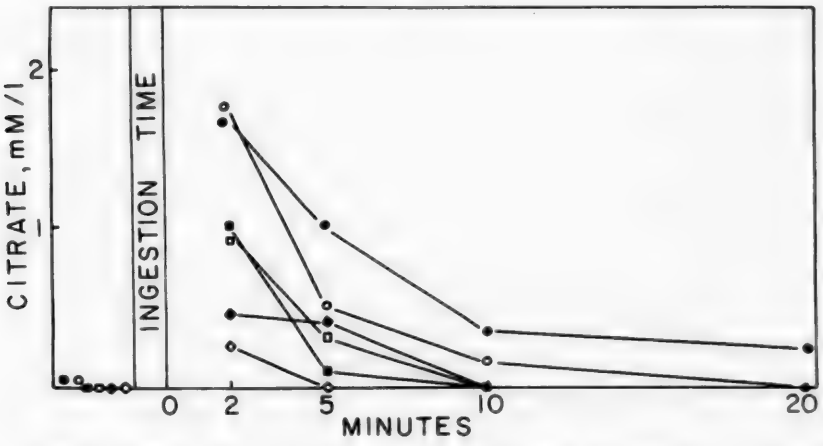

Fig. 1.—Salivary citrate after orange ingestion. Fasting values of Subjects 1 to 6 appear in this order from left to right at lower left corner.

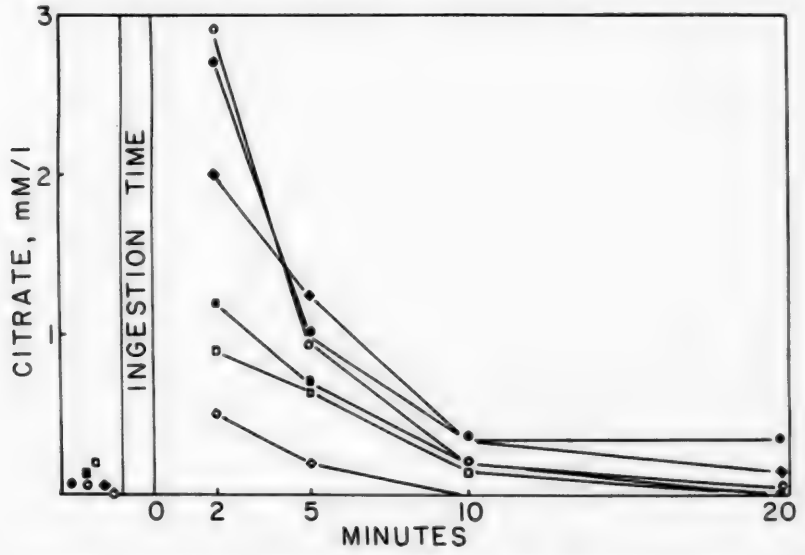

Fig. 2.—Salivary citrate after grapefruit juice ingestion. The same designations as in Fig. 1.

Results.—The saliva of six healthy adult subjects was examined before and after they had ingested each of the citric acid sources. The results appear in Figs. 1 and 2. The fasting values are of the order found by Zipkin and

*This modification enabled accurate analysis down to 0.25 mM per liter with 0.25 ml. samples. Ten consecutive determinations of a 2 mM citrate solution gave the standard deviation 0.099 mM per liter. A detailed description of the procedure may be obtained from the author.

McClure in stimulated saliva.[31] An increased salivary citrate content is found during the postingestion period. After 10 minutes the level has again approached the fasting value, although in one case an elevated level was still found after 20 minutes. This subject was tested repeatedly with consistently high postingestion results. It may be of interest to note that (1) the citrate contents found were higher after drinking grapefruit juice than after taking orange; (2) the highest and the lowest individual curves were obtained with the same subjects in both tests; and (3) during the collections it was noticed that Subject 2 had an extraordinarily rapid secretion and Subject 6 a rather slow secretion, that is, the reversed order to the rate of disappearance of the citrate.

These curves bear a strong resemblance to those obtained for total and reducing sugar in the saliva after carbohydrate ingestion,[6, 18] indicating that the mechanism involved may be the same in both cases, namely, an intraoral retention. To test this supposition, salivary citrate analyses were performed in the standard way with Subjects 1 and 2 after they had swallowed large amounts of citric acid in capsules. Five hundred milligrams of citric acid was given in two or three thin gelatin capsules during the 1-minute period. Although this quantity is several times that contained in the whole blood volume, all the saliva analyses up to 20 minutes were below the lower limit of the method. It may be pointed out that there are previous reports that the serum citrate level is hardly increased by even greater peroral doses.[25, 26]

Thus, it can be concluded that the increased salivary citrate content after ingestion depends on the retention in the mouth of the citrate of the food.

2. THE ENAMEL APATITE SOLUBILITY IN THE SALIVA IN THE PRESENCE OF CITRATE

The solubility of the calcium phosphate of dental enamel has been found to deviate strongly from that predicted from established solubility products.[5, 9] At pH values above 5 the solubility is much greater in saliva than, for instance, in thermodynamically comparable NaCl solutions. This result probably is due mainly to the presence of un-ionized calcium and phosphate complexes in saliva. Since citrate is known to be a strong calcium-combining substance, its presence in the saliva might theoretically be supposed to increase further the solubility of calcium salts.

The biologic interest in calcium citrate complexes seems to have been considerably resuscitated, to judge from the publication of several articles on this subject in later years.[12, 14, 28, 29] Although still somewhat controversial, the recent work has provided increased knowledge in this field. With the normal salivary citrate level below 0.1 mM per liter,[31] no great significance is to be attached to the effect of the ion on the solubility of calcium salts. However, if the degree of ionization of the complex Ca Cit is calculated according to Heinz[12] for a 1 mM concentration, $\alpha = 0.11$ is obtained; the same calculation for (Ca H Cit) at the same concentration gives $\alpha = 0.50$. With the normal salivary Ca concentration of about 1.5 mM per liter and with the citrate levels found after citrus fruit ingestion, a complex formation of significant influence on the solubility of the calcium salts is thus conceivable.

This concept seems to be further supported by the experimental findings, mentioned in the introduction, of erosion caused by citrates, even at nearly neutral reaction.

The experiments reported in this section were designed to test this possibility. Saliva was equilibrated with powdered dental enamel at 37° C., the same saliva being tested with and without added citric acid in each experiment. After attained equilibrium, the analyses necessary for estimation of the calcium phosphate solubility were made: pH, calcium, inorganic phosphorus, and ionic strength. Calculations of the solubility were based on Bjerrum's formulas and values for hydroxy apatite,[2] since this is the most stable calcium phosphate and is also the dominating phosphate in the enamel. A more complete description of the procedure follows:

Methods.—For each experiment 60 ml. of my paraffin-stimulated saliva was used. Thirty milligrams of powdered thymol were dissolved in the saliva in order to prevent bacterial decomposition of the citrate and other organic constituents. It had been ascertained in advance that thymol in this concentration did not affect the subsequent analytic procedures. The saliva was then divided equally into two portions, A and B. To A was added enough citric acid to give a concentration of 1.5 to 3.0 mM per liter. A and B were then divided equally into A1, A2, B1, and B2 and to each was added 40 mg. of powdered human enamel which had been purified according to the method of Brekhus and Armstrong[3] and passed through a hundred-mesh sieve. To A2, B1, and B2 were then added varying amounts, up to 0.4 ml., of *N* HCl. The purpose of the hydrochloric acid was to produce a pH value, after equilibration, of 7.0 to 7.5 for A1 and B1 and about 5.5 for A2 and B2. Citrate-containing saliva was thus compared with the same saliva without added citrate at these two pH levels, of which one corresponds to the original reaction of the saliva and the other to about the critical level where dissolution of the enamel apatite begins.

All four saliva-enamel mixtures were then equilibrated at 37° C. for the same time, varying between 19 and 23 hours in four experiments. In two experiments the mixtures were stirred continuously with 5 per cent CO_2 in O_2; the gas was first saturated with water and warmed to 37° C. by passing it through a 0.05 *M* NaCl solution of this temperature. In the other two experiments the saliva-enamel mixtures were shaken in small stoppered bottles without special measures to maintain the CO_2 pressure.

After equilibration, the pH value was first measured with a Beckman pH-meter, model H 2. The mixtures were centrifuged for 5 minutes at 2,000 r.p.m., and the supernatant liquids cautiously decanted. The resistance to a 2,000-cycle alternating current was measured, followed by pipettings of the samples for analyses of citrate, calcium, and phosphorus. About 45 minutes was required for these measures.

The ionic strength was estimated from the electric resistance, compared with that of a 0.050 *M* NaCl solution, according to a method described previously.[5] Citrate was determined with the technic described in Section 1 of this article. Calcium was determined according to methods described in a separate

article.[7] These involve titration of Ca + Mg with disodium ethylenediaminetetraäcetic acid, using Eriochrome Black T as indicator, and deduction of Mg as determined by a Titan Yellow technic.

Inorganic phosphorus was determined according to a previously described colorimetric technic.[5] For this determination the centrifugate after precipitation at the isoelectric pH of saliva was used.[1] This was attained by addition of 0.3 ml. 0.1 *N* HCl to 1 ml. saliva, which gives a much clearer supernatant liquid than, for instance, precipitation with trichloroacetic acid.

The relative solubility n, that is, the ratio *solubility found: solubility expected from Bjerrum's formulas,* was obtained by inserting the analytic values in the formula:

$$\log n = \frac{7.5 - 16.5 \sqrt{\mu} - 5p\,[Ca] - 3p\,[HPO_4] + 4\,pH}{9}, \qquad (a)$$

where μ = ionic strength,
p = negative logarithm of the following ionic concentration.
The term p $[HPO_4]$ was obtained from the formula:

$$p\,[HPO_4] = p\,[P] + \frac{\log K'' + pH}{K''}, \qquad (b)$$

where K″ = second "incomplete" dissociation constant of H_3PO_4.

The derivation of these formulas is given in a previous work[5] and a convenient nomogram for part of the calculation has been presented.[8] All the calculations were based on the assumption that equilibrium was attained from the undersaturated side, which seemed probable on account of the addition of citric and hydrochloric acid to the previously shaken salivas. If, at the highest pH values, equilibrium was attained instead from the supersaturated side, the first term in the numerator of equation (a) would be 6.5 and the log n values would be lowered by 0.11. This would affect both citrate experiments and controls to the same extent and would not change the comparisons appreciably.

RESULTS

The results of analysis and calculation are given in Tables I, II, III, and IV, where all concentration figures denote millimoles per liter. The relative solubilities found are represented logarithmically in Fig. 3.

It appears from the diagram that the points have fallen roughly along an oblique line intersecting the zero line at a pH value somewhat above 5. This

TABLE I

NO Co_2 BUBBLING

EXPERIMENT	CITRATE	pH	Ca	P	μ	LOG n	n	DIAGRAM SYMBOLS
A1	1.65	7.48	0.72	3.50	0.064	1.10	12.6	⊙
A2	1.62	5.40	3.61	4.90	0.080	0.108	1.28	⊙
B1	<0.1	6.83	0.83	3.40	0.068	0.75	5.6	○
B2	0.0	4.95	3.01	4.75	0.074	−0.27	0.54	○

TABLE II

NO Co_2 BUBBLING

EXPERIMENT	CITRATE	pH	Ca	P	μ	LOG n	n	DIAGRAM SYMBOLS
A1	1.87	7.55	1.38	4.50	0.056	1.36	12.3	⊡
A2	1.87	4.98	3.36	6.80	0.059	−0.12	0.76	⊡
B1	0.17	7.25	1.14	4.10	0.055	1.14	13.8	□
B2	0.22	5.60	1.79	5.40	0.059	0.17	1.48	□

TABLE III

WITH Co_2 BUBBLING

EXPERIMENT	CITRATE	pH	Ca	P	μ	LOG n	n	DIAGRAM SYMBOLS
A1	1.65	7.10	1.14	3.80	0.055	1.05	11.2	△
A2	1.47	5.65	1.86	4.60	0.057	0.20	1.58	▲
B1	<0.1	7.15	0.70	3.45	0.053	0.96	9.1	△
B2	0.15	5.65	4.31	6.10	0.059	0.44	2.76	△

TABLE IV

WITH Co_2 BUBBLING

EXPERIMENT	CITRATE	pH	Ca	P	μ	LOG n	n	DIAGRAM SYMBOLS
A1	3.00	7.20	1.12	4.15	0.051	1.13	13.4	▽
A2	3.00	5.60	1.97	5.10	0.055	0.20	1.58	▽
B1	<0.1	7.25	0.64	3.90	0.055	1.03	10.7	▽
B2	0.2	6.10	3.32	5.55	0.060	0.69	4.90	▽

result is in accordance with previous findings. For example, at pH 7.0 log n is about 1.0, that is, n = about 10, indicating a ten times higher solubility in the saliva than in a CO_2-free salt solution of the same pH, temperature, and ionic strength. No significant difference can be found between experiments with and without CO_2 bubbling.

Experiments with and without citrate show no clearly different trend. This implies that the calcium-combining capacity of these citrate concentrations is of no significance to the calcium phosphate solubility in this clinically important pH region.

DISCUSSION

Of the results, the most interesting finding is that of an unchanged calcium phosphate solubility in the saliva in the presence of up to 3 mM citrate per liter. Two hypothetic explanations may be offered. First, the noncitrate complex formation in the saliva may be so extensive that addition of these citrate concentrations does not produce any detectable difference with the applied methods. This point should be especially valid at the higher pH levels where the relative solubility reaches its highest values.[5] Second, there are many indications of special solubility conditions of a little understood nature for the system calcium-citrate-phosphate: a strongly variable citrate precipitability with varying calcium to phosphate ratios;[15] the presence of phosphate in all investigated citrate-

containing concrements from the animal kingdom;[27] varying effects on resorption, blood calcium level, and ossification with varying calcium to phosphate to citrate ratios in animal experiments and clinical applications.[4, 11, 28-30] The need for further research into this problem is obvious.

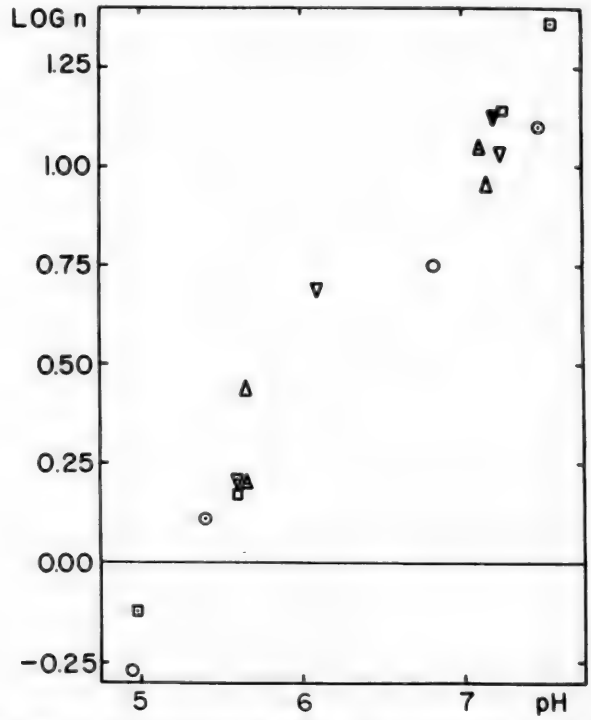

Fig. 3.—Solubility of enamel apatite in saliva with and without added citric acid. See text and Table I.

The experimental results seem to justify the clinically important inference that no prolonged solubility-increasing effect is to be expected from ingestion of even the most citrate-rich common foods and beverages. Nor can the citric acid concentrations found after 5 minutes or later overcome the average buffering of the saliva,[5] as is easily calculated. The observed erosive effects of these fruits and beverages, therefore, should be practically limited to the time of ingestion.

SUMMARY

The citrate content of the saliva was increased after ingestion of citrate-rich fruits and beverages, due to an intraoral retention. The concentration decreased rapidly, being only slightly above the fasting value after 10 minutes.

Citrate concentrations of the order occurring 2 minutes after the test ingestions had no apparent influence on the solubility of the enamel apatite in the saliva, as tested in equilibrium experiments in the pH range 5 to 7.5.

I wish to acknowledge my great indebtedness to several members of the staff of each of the institutions of origin for invaluable help and suggestions regarding methods and instrumentation, especially to Drs. Frank J. McClure and Isadore Zipkin, of Bethesda, Md., and to Drs. Wallace D. Armstrong and Leon Singer, of Minneapolis, Minn.

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DEMINERALIZATION OF HARD TISSUES BY ORGANIC CHELATING AGENTS AT NEUTRAL pH

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NUMEROUS methods have been introduced for decalcifying hard tissues for purposes of histologic and histochemical studies. These methods have been reviewed by Ham and Harris[2] and may be listed as follows: (1) Decalcification with weak solutions of strong acids such as nitric or hydrochloric acid. (2) Decalcification with strong acids plus phloroglucin. (3) The use of ordinary fixing agents such as Bouin's fluid or formic acid, which also serve as decalcifying agents. (4) The use of neutral salts of magnesium citrate. (5) The acid-extraction method using 13 per cent sodium citrate solution in 45 per cent formic acid as described by Morse.[4] (6) Electrolytic method. (7) Decalcification by the use of trifluoroacetic acid.[7] (8) Dotti, Paparo, and Clarke[1] have employed an ion exchange resin and formic acid with satisfactory results. The resin replaced the sodium citrate as a means of removing the calcium ions from solution. It will be observed that with the exception of the magnesium citrate method all decalcifying agents employ low pH's as a method of bringing bone salts into solution.

The recent introduction of a powerful organic chelating agent, ethylenediamine tetracetic acid (abbreviated in this article as Edt), suggested a possible method of demineralizing hard tissues with alkaline agents. Preliminary reports[5, 9] indicated that reasonably rapid demineralization of hard tissues with solutions of Edt was possible. This article reviews the chemical properties of Edt and reports on experiments designed to determine the best method of using this agent for routine decalcification of hard tissues.

PROPERTIES OF ETHYLENEDIAMINE TETRACETIC ACID

The sodium salts of ethylenediamine tetracetic acid are noncolloidal organic chelating agents that resemble the inorganic polyphosphates (e.g., sodium hexametaphosphate) in their ability to form soluble nonionic chelates with a

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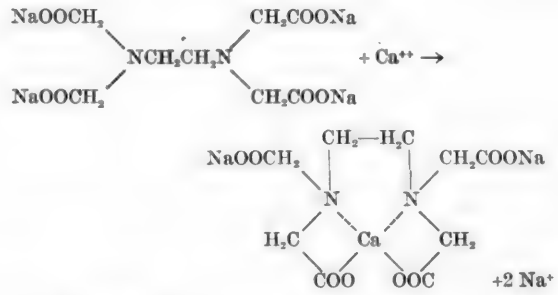
large number of metallic ions. The reaction with calcium is thought to proceed as follows:



Experimentally it may be observed that a considerable drop in pH occurs when a neutral calcium salt such as calcium chloride is added to a solution of the disodium salt of Edt. This is due to the liberation of hydrogen ions in the course of complex formation:

$$Ca^{++} + Na_2H_2Edt \rightleftharpoons Na_2(CaEdt) + 2\ H^+$$

This phenomenon makes it necessary to buffer Edt solutions where large amounts of calcium are to be chelated and where drops in pH are undesirable.

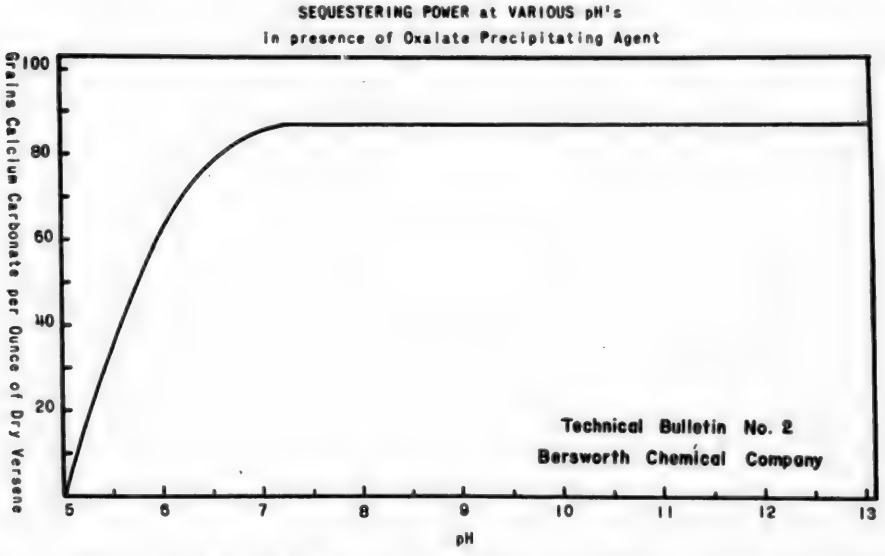


Fig. 1.—Effect of pH on the chelation of calcium by ethylenediamine tetracetic acid (from Technical Bulletin No. 2, Bersworth Chemical Company).

The effect of pH on the chelating power of Edt toward calcium is illustrated in Fig. 1. It is noted that calcium is strongly chelated above pH 6 and that the curve levels off above pH 7.5. The efficiency drops markedly below pH 6. This property of Edt makes it valuable as a demineralizing agent.

Edt is soluble to the extent of about 0.03 per cent at room temperature. The disodium dihydrogen salt is soluble to the extent of 6 per cent. The tri- and tetra-sodium salts are very soluble and dissolve to the extent of 30 and 50 per cent, respectively, at room temperature.

The negative logarithms of the dissociation constants are reported by Schwarzenbach and Ackermann[8] to be 2.0, 2.67, 6.16, and 10.26 at 20° C. for the first, second, third, and fourth protons, respectively. The reader is referred to their paper for a more complete account of the equilibriums between the combining ions.

MATERIAL AND METHODS

The acid and sodium salt of Edt was obtained from the Bersworth Chemical Company, Framingham, Mass., under the trade name of Versene. The acid salt, obtained in 100 per cent dry powder, was neutralized to desirable pH's by using solutions of sodium hydroxide of suitable strength. The sodium salt of Edt, obtainable in 34 per cent liquid form, was adjusted to desirable pH's by the use of hydrochloric or citric acids.

The ability of Edt to demineralize hard tissues was determined by its action on the femurs, tibias, and mandibles of 120-day-old littermate rats. These bones were stripped of all external soft tissue, and fixed in 10 per cent formalin prior to decalcification with Edt. The individual bones differ in chemical composition and are not quantitatively comparable. However, when bones obtained from different rats of the same litter and age were placed in identical solutions of Edt, the demineralization rate was approximately the same. Hence, it was possible to quantitate roughly the results obtained. Each experiment was run in duplicate and the results reported were readily reproducible.

The rate of demineralization was determined by x-raying the specimens at different intervals. The absence of radio-opacity was taken as evidence of complete demineralization. The use of x-rays for assessing the rate of demineralization, though satisfactory, is only roughly quantitative.

All specimens used for demineralization, unless stated otherwise, were suspended near the top of a column of 500 ml. of the demineralizing solution at room temperature.

EXPERIMENTAL FINDINGS

(1) *Effect of pH on the Rate of Demineralization of Bone Specimens by Edt.*—Four rat mandibles and four femurs, previously fixed in 10 per cent formalin, were suspended in 500 ml. of 0.25 M. of Edt solution, 25° C. at pH's of 6.02, 7.49, 9.53, and 12.01. Table I summarizes the results obtained.

TABLE I

TIME REQUIRED FOR COMPLETE DEMINERALIZATION OF RAT MANDIBLES SUSPENDED IN 500 ML. OF 0.25 M. EDT AT VARYING pH'S, 25° C.

pH	6.02	7.49	9.53	12.01
Days required for demineralization	8	9	16	18
pH after demineralization	6.03	7.49	9.50	11.77

Fig. 2 shows a roentgenogram of rat mandibles taken seven days after the specimens were placed in solutions of Edt at pH's of 6.02, 7.49, 9.53, and 12.01. The bone specimens suspended in solutions of pH 6.02 and 7.49 are almost completely decalcified; the bone specimens suspended in solutions of pH 9.53 and

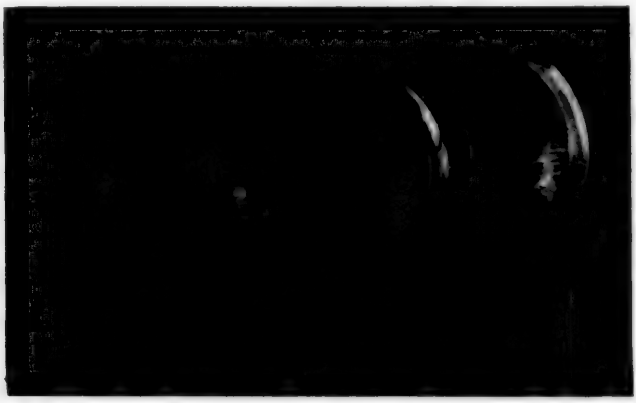


Fig. 2.—Roentgenograms of rat mandibles seven days after specimens were suspended in solutions of Edt at pH's 6.02, 7.49, 9.53, and 12.01 from left to right respectively.

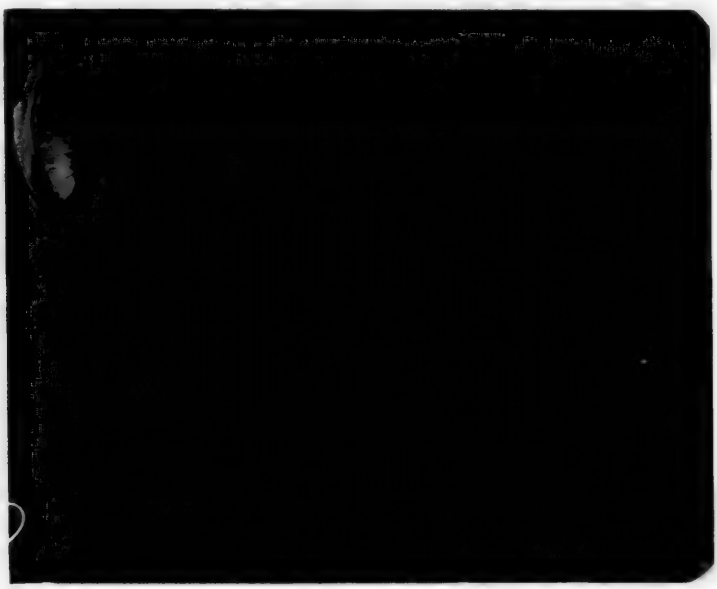


Fig. 3.—Roentgenograms of rat mandibles taken seven days after suspension in solution of Edt at pH 7.43 at room temperature. From left to right, the upper and lower radiograms represent bone specimens that were placed in 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, and 0.9 M. solution of Edt, respectively.

12.01 remain rather highly calcified. The optimum pH for demineralization of bone specimens by Edt is around the neutral point. These results do not parallel the curve in Fig. 1 for reasons discussed later.

(2) *Effect of Concentration of Edt on the Rate of Demineralization of Bone Specimens.*—Rat mandibles, weighing approximately 0.40 Gm., were suspended in solutions of Edt, pH 7.43, at concentrations varying from 0.1 to 0.9 M. One week later the specimens were x-rayed to determine the extent of demineralization (Fig. 3). The length of time required for complete demineralization of bone specimens shown in Fig. 3, is represented graphically in Fig. 4. The experiment indicated that increasing the concentration of Edt from 0.1 M. to 0.5 M. results in a discernible increase in the velocity of demineralization of the bone specimens. Increasing the concentration of Edt beyond 0.5 M. does not appreciably alter the speed of demineralization of small bone specimens.

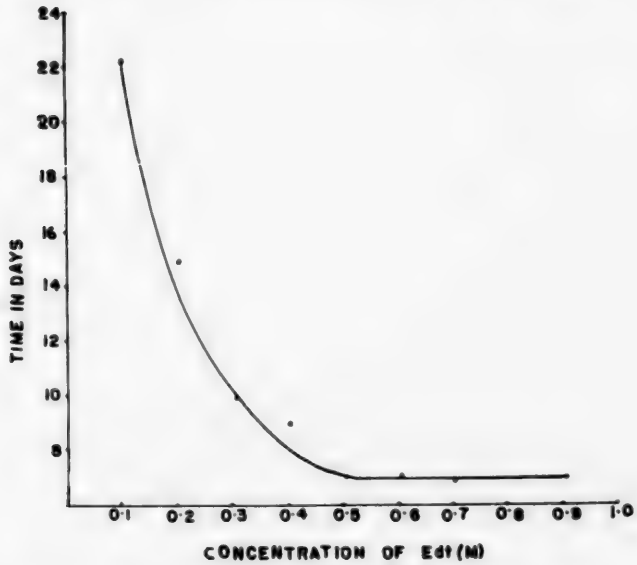


Fig. 4.—Graph to indicate the effect of concentration of Edt on velocity of demineralization of bone specimens (rat femurs) weighing approximately 0.40 Gm. at pH 7.40 at room temperature.

(3) *Comparison of Rate of Demineralization of Bone Specimens by Edt at Various Temperatures and With Other Decalcifying Agents.*—According to Schwarzenbach and Ackermann,[8] the complex of Edt and calcium is stable over a wide range of temperature. It was important, therefore, to determine whether the rate of demineralization of bone tissue is significantly increased by elevating the temperature of the Edt solutions. Rat femurs were suspended in solutions of Edt, 0.5 M., pH 7.49, at temperatures of 4°, 25°, 37.5°, and 60° C. Table II indicates that demineralization of bone specimens is greatly increased by elevating the temperature of the solutions. This result has important practical connotations.

A comparison of the rate of demineralization of bone specimens by various decalcifying agents (Table II) indicates that at 25° C. solutions of Edt are approximately one-seventh as effective as a 5 per cent solution of nitric acid;

however, at a temperature of 60° C. the solution of Edt decalcifies almost as rapidly as a 5 per cent solution of nitric acid at room temperature. Solutions of Edt decalcify bone at approximately one-half the speed required by solutions of formic acid-sodium citrate. Solutions of Edt are much more effective than neutral solutions of magnesium citrate for demineralization of bone.

TABLE II

COMPARISON OF RATE OF DEMINERALIZATION OF BONE SPECIMENS BY EDT, pH 7.49, 0.5 M., AT VARIOUS TEMPERATURES AND WITH OTHER DECALCIFYING SOLUTIONS OF EQUAL VOLUME

DEMINERALIZING SOLUTION	EDT	EDT	EDT	EDT	5% HNO_3	FORMIC CITRATE	FORMIC RESIN	MAGNESIUM CITRATE
Temperature	4° C.	25° C.	37.5° C.	60° C.	25° C.	25° C.	25° C.	25° C.
Approximate days for complete demineralization	10	7	3	1	1	3	1	35

(4) *Additions of Accelerators to Edt and Their Effect on the Rate of Demineralization.*—Hahn and Reygadas[3] have reported that the neutralization of Edt by sodium pyrophosphates instead of sodium hydroxide speeds decalcification of "iron containing tissue." Dotti, Paparo, and Clarke[1] employed exchange resins together with formic acid to increase the velocity of demineralization of tissues. Sodium citrate has been used to remove ionic calcium from solutions. These reagents were tested in order to determine their effect on the velocity of the demineralization of bone by Edt.

A solution of Edt, 0.15 M., pH 7.4, was prepared by neutralizing the acid salt with sodium pyrophosphate. This solution decalcified rat femurs and rat mandibles approximately 2 to 4 days sooner than a control solution of the same molarity, and neutralized to a pH of 7.4 by sodium hydroxide.

The rate of demineralization of a solution of Edt neutralized with citric acid was not significantly different when compared with a solution neutralized with hydrochloric acid to a similar pH.

The addition of a resin (ammonium salt of a sulfonated resin) to Versene at neutral pH's did not increase the velocity of demineralization of bone specimens.

(5) *Effect of Suspending Bone Specimens at Different Heights in a Column of Edt.*—Hahn and Reygadas[3] stated that mother-of-pearl buttons decalcified faster when the specimens were suspended near the top of the column of the solution. A similar experiment with bone specimens indicated that demineralization of bone specimens placed at the bottom of a vessel is much slower than demineralization of similar specimens suspended several inches from the bottom. Raising the specimen further did not appreciably increase the velocity of demineralization.

DISCUSSION

The ability of ethylenediamine tetra acetate to form complexes with calcium salts at neutral and alkaline pH's offers a new and satisfactory method of de-

mineralizing hard tissues without subjecting the tissue specimens to low pH's and concomitant drastic denaturation of protein.

Demineralization of hard tissues by Edt is best explained by the principle of the solubility product constant. When a substance of low solubility such as a bone or a tooth is placed into some water, a small but very definite amount of this substance dissolves. For didactic purposes, it may be assumed that the inorganic complex of bone is predominantly $Ca_3(PO_4)_2$. When placed in water a definite amount of calcium and phosphate will dissolve, until the bone is in equilibrium with its saturated solution. The product of the molar concentrations of the calcium and phosphate ions must equal a constant at a given temperature. The constant denoted by the letter "K" is the solubility product constant of bone. If now Edt is added, some calcium ions are withdrawn from the solution. More bone substance will dissolve to satisfy the solubility product constant. The equilibrium thus is shifted to the right, as illustrated by the following equations, until the bone specimen is completely dissolved.

$$\text{Bone} \rightleftharpoons (Ca)^2 \times (PO_4)^3 = K$$

$$+$$

$$\text{Edt}$$

$$\downarrow$$

$$\text{CaEdt}$$

This principle also explains the apparent discrepancy between the optimal pH of Edt for chelation of calcium carbonate and for demineralization of hard tissues. Fig. 1 illustrates that Edt chelates calcium best at alkaline pH's, and that the curve flattens above pH 7.5. However, at pH's above 10.5 the rate of demineralization of bone specimens by Edt is slower than at neutral pH's. This interesting fact is explained by the observation that the solubility product constant of bone decreases as the pH increases. With fewer calcium ions available at a pH >10 than <10, the rate of demineralization is not as rapid.

Experimental findings (Table I) indicate that a pH of 7 to 8 is suitable for demineralizing hard tissue specimens. A concentration of 0.5 M. of Edt is sufficient for specimens weighing 0.5 ± 0.1 Gm. The chelating capacity of Edt is so great that only a fraction of available Edt is used up during demineralization of small specimens. Thus, for small bone specimens (up to 1 Gm.), changing solutions is unnecessary. Experimental findings also suggest that the use of sodium pyrophosphate is valuable in increasing the velocity of demineralization of large bone specimens. Increase of temperature speeds demineralization markedly, and may be used where rapid demineralization is required. Specimens should be suspended near the top of the liquid. The complex-containing solution, having a higher density, sinks, bringing about an automatic circulation and renovation of the reagent.[3]

The greatest disadvantage to Edt lies in the fact that its rate of demineralization is slow when compared with acid-decalcifying solutions. Bone specimens weighing approximately 0.4 Gm. may be decalcified by 5 per cent nitric acid in one day. A comparable specimen may require two to three days with formic-citric acid; five to eight days with Edt at pH 7.4 at 25° C.; one day with formic acid-resin; thirty days when a neutral solution of magnesium citrate is used.

Thus, Edt solutions have approximately one-seventh the velocity of demineralization of acid solutions; on the other hand, they are about seven times as fast as any existing neutral solution used for demineralization of hard tissues.

The use of Edt for demineralization purposes should prove useful in histochemical, chemical, and histologic studies of small specimens of calcified tissues. The action of Edt solutions is very mild as contrasted with acid solutions. The protein fraction is not as easily destroyed and the development of CO_2 bubbles is prevented. This suggests that enamel protein may be more easily preserved and recovered by demineralizing with Edt than by using previously described methods. That enamel protein may be readily preserved is illustrated in Fig. 5. The tooth depicted was suspended in a 0.5 M. Edt solution at pH 7.3 for four weeks. The mineral salts of the enamel dissolved, leaving a protein envelope intact. Demineralization of enamel is much faster than that of dentin. Penetrability and surface area are important factors in determining speed of demineralization.

Fig. 5.—Photograph of tooth specimen suspended in Edt, 0.5 M., pH 7.3 at 25° C. for four weeks. Note the preservation of enamel protein.

SUMMARY

(1) A new method for demineralizing calcified tissues at neutral pH's is described. The demineralizing agent is an organic chelating agent, ethylenediamine tetracetic acid.

(2) The chemical properties and the experimental findings relative to the use of this agent as a decalcifying agent are reported.

(3) The acid powder neutralized to a pH of approximately 7.4 by the use of sodium hydroxide, or the sodium salt of ethylenediamine tetracetic acid neutralized to the same pH by hydrochloric acid, in a concentration of 0.5 M., at room temperature, makes a suitable demineralizing medium for small specimens (weighing about 0.5 Gm.) of bone tissue.

(4) The use of higher temperatures greatly increases the speed of demineralization of bone specimens by Edt.

(5) Edt solutions are useful at a neutral pH; hence, they are mild demineralizing agents. This method should prove useful in histologic, histochemical, and chemical studies of bone tissues.

(6) Edt solutions demineralize bone specimens at a rate about one-seventh that of acid solutions and about seven times as rapidly as a neutral decalcifying solution of magnesium citrate.

(7) The mechanism of action of Edt as a demineralizing agent is described.

The technical assistance of Miss Sybil Vail and the preparation of illustrations by Mr. G. H. Loft are acknowledged.

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AN EFFICIENT WATER SYSTEM FOR SMALL LABORATORY ANIMALS

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OVER a period of approximately ten years I have often noticed small laboratory animals without an efficiently functioning water system. In the study of dental caries in such animals as hamsters and rats, inadequate water supply may place considerable bias on a group. The system to be described has been used very successfully over the past several years.

Factors which add to the problem of water supply include: (a) improperly designed water tubes and poor attachment of water bottles, (b) dislocation of tubes and bottles by animals playing with the system, (c) inadequate supervision by laboratory assistants and animal caretakers. Because this problem was so disturbing in the course of studies on experimental dental caries in hamsters, a water system has been developed which has eliminated almost all cause for concern.

Drinking tubes are made of regular or Pyrex glass with an outside diameter of 10 mm. Tubes of smaller diameter should not be used. Although tube length might be varied somewhat to accommodate a particular bottle arrangement, in this laboratory tubes are cut about 4½ inches in length. Each tube is bent in the middle at an angle of approximately forty degrees (Fig. 1, *A*). Proper angle of the tube will facilitate rapid exchange of air and water, will completely prevent air traps, and will be conducive to rigidity of the entire system, thus minimizing its displacement by the animals. With tubes bent at this angle it is generally necessary to enlarge slightly one opening in the mesh of the screen of the cage by inserting a metal rod into one mesh and bending upward to approximate the angle of the tube. Then the hole will not be overenlarged and will accommodate the tube adequately, yet with minimal play.

Aperture and shape of the tube opening at the drinking end is critical. An aperture of approximately 4 to 5 mm. is recommended. This opening is produced by flaming the end of the tube until it is somewhat smaller than desired. By means of a fine carborundum stone mounted on a motor, the flamed end of the tube is ground, with ample water as a lubricant, until the aperture is 4 to 5 mm. wide (Fig. 1, *A* and *B*). Small mounted diamond stones ½ inch in diameter are excellent for this purpose and are available through the regular dental supply houses at reasonable cost. If diamond stones are used, cutting should be done under water with very light pressure and high speed.

Grinding is believed to be well worth the extra effort involved, as it insures the correct aperture of the drinking end of the tube and reduces surface tension at the lip, thus facilitating release of water to the animal and air to the bottle. A properly ground tube will not leak but will often have a free drop of

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water hanging at the lip. The outside edge of the tube end can be beveled on the edge of the stone in a few seconds. It is not necessary to polish or to burnish the ground ends by heat. The edges are not sufficiently rough or sharp to injure the animals' tongues.

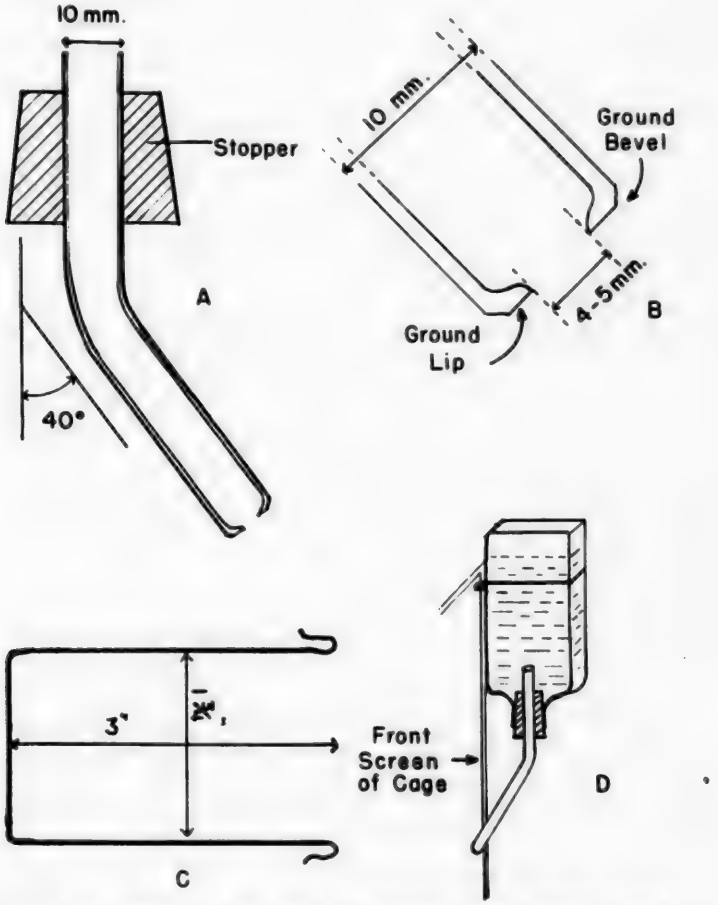

Fig. 1.—*A*, Cross section of water tube and stopper showing angle of tube and correct position of stopper. *B*, Enlarged cross section of drinking end of tube showing dimensions and approximate shape of ground lip. *C*, Shape and dimensions of wires used to attach bottles to cages. *D*, Position of bottles and tubes on cages.

Stopper-tube relations: The bottle end of the tube should always be long enough to pass completely through the stopper (Fig. 1, *A* and *D*). It is not desirable to have the bottle end of tubes terminate within holes of the stoppers. Not only may the aperture be reduced by the size of the hole in the stopper so as to cause improper air-water exchange, but also fragments of stopper and occasionally other agents may occlude the opening within the stopper itself and pass unnoticed for a considerable period of time.

Bottle arrangement can be standardized so that service is easy, animals are prevented from nibbling stoppers and displacing the tubes, and checking of an entire rack is possible by a single quick inspection. In this laboratory, amber eight-ounce bottles (Armstrong's Blakes Wide Mouth) are used, although bottles of this type are not imperative. Attachment wires for holding bottles on cages should be bent carefully. Our wires are made from pieces of iron baling wire salvaged from bales of shavings and somewhat similar to that used in coat hangers. The design is shown in Fig. 1, *C*. The wire is first attached to the cage, and then the bottle is slipped into place. If the dimensions of the wire are correct, for example, 3 inches by 1¾ inches, bottles will be held snugly in place with just adequate play for removing without difficulty. Wires can be removed and reattached if desired.

With tubes designed and bottles arranged as described, an entire rack of cages can be quickly inspected merely by shaking the rack and observing the movement of the water level in the bottles.

This arrangement has also been found satisfactory for supplying water to animals in breeding cages. The position and height of water tubes is critical when inserted into a cage with shavings and cages with females and litters. The end of the tube should not be so low that it touches the shavings lest they act as a wick, causing release of all the water and flooding of the cage, yet the tube should be low enough so that young animals can get to it. It is now our policy to place two water bottles on all cages with young animals as a precautionary measure.

When tubes and bottles are kept clean and full, the system described will almost entirely eliminate problems associated with supplying water to small animals kept in cages.

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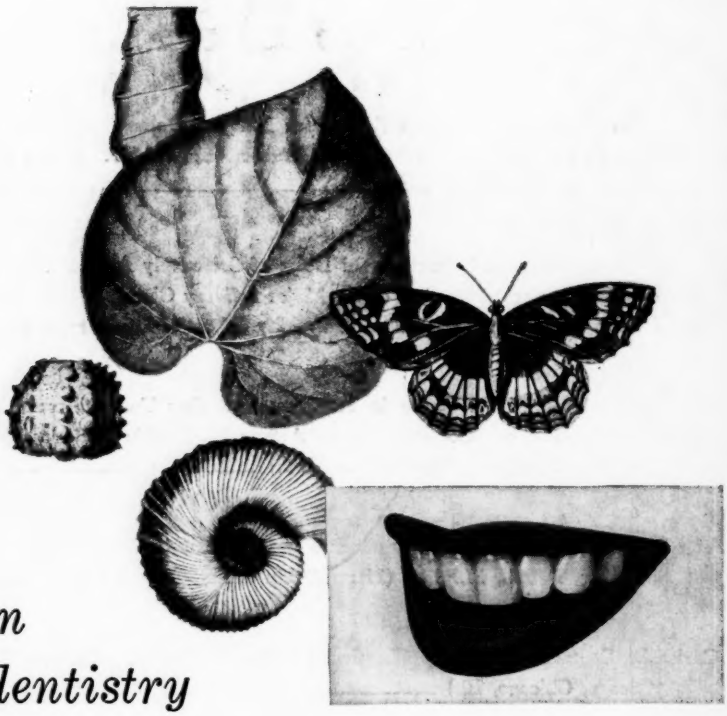

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